Supplementary file

Nitric oxide-soluble guanylyl cyclase pathway
as a contributor to age-related memory impairment in *Drosophila*

Ayako Tonoki¹, Saki Nagai¹, Zhihua Yu¹, Tong Yue¹, Sizhe Lyu¹, Xue Hou¹, Kotomi Onuki¹,
Kaho Yabana¹, Hiroki Takahashi², Motoyuki Itoh¹

1. Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 260-8675, Japan
2. Medical Mycology Research Center, Chiba University

Corresponding Author: Ayako Tonoki
Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 260-8675, Japan
Tel: +81-43-226-2890
Fax: +81-43-226-2890
E-mail: tonoki@chiba-u.jp
Supplementary information

Figure S1. Neuron-specific knockdown of gycβ enhances intermediate-term memory
(Related to Figure 1).

(A, B) Venn diagrams showing the overlap between two different gene lists of RNA-sequencing data and genome-wide RNAi screen data: four genes are negative memory regulators with age-dependent upregulation of gene expression (A) and fifty-four genes are positive memory regulators with age-dependent downregulation of gene expression (B). (C) Each candidate gene was transiently knocked-down in neurons only after the flies were fed RU486 (RU) using Elav-GS. When 5-day-old flies were fed RU-containing food for 5 days, memory performance was tested at 3 hr after conditioning. Enhancements in 3-hr memory were observed in flies expressing cp190RNAi or gycβRNAi in neurons compared with control. (n = 18, 18, 18, 18, 10, 10, 10, 12, and 12 for control RU-, control RU+, cp190RNAi RU-, cp190RNAi RU+, GycβRNAi RU-, GycβRNAi RU+, amonRNAi RU-, amonRNAi RU+, SA RNAi RU-, and SA RNAi RU+ respectively. Two-way ANOVA $F_{(4, 127)} = 8.882, p < 0.0001$ for row factor and $F_{(4, 127)} = 10.13, p = 0.0018$ for column factor. post hoc Tukey’s multiple comparisons test, *$p < 0.05$, cp190RNAi RU- versus cp190RNAi RU+; **$p < 0.05$, control RU-/RU+ versus GycβRNAi RU+.)
Data are mean ± SEM for (C).

**Figure S2. Neuron-specific knockdown of gycβ (Related to Figure 1).**

(A) qPCR shows that the silencing efficiency of the knockdown of gycβ genes in neurons was approximately 50% in the flies expressing gycβ^{RNAi-HMJ22589} (gycβ^{RNAi}). (n = 3, 3, 3, and 2 for control RU-, control RU+, Gycβ^{RNAi} RU-, and Gycβ^{RNAi} RU+ respectively. Two-way ANOVA $F_{(1, 7)} = 16.57$, $p = 0.0047$, post hoc Tukey’s multiple comparisons test, *$p = 0.0320$, control RU+ versus RNAi RU+). (B) Shock avoidance test in gycβ knockdown flies. There was no significant difference between the experimental group and the control group in the avoidance of electric shock (n=10 for each data. $t_{(18)} = 0.2334$, $p = 0.8181$). (C) Odor avoidance test in gycβ knockdown flies. There was also no significant difference between the experimental group and the control group in the avoidance of odors, Oct and Benz (n=10 for each data. Two-way ANOVA $F_{(1, 36)} = 2.55$, $p = 0.1191$). (D) 3-hr memory after cold shock treatment given 2 hr after conditioning. Transient and neuron-specific knockdown of gycβ (Elav-GS> gycβ^{RNAi}) significantly enhanced 3-hr memory after cold shock compared to the control group (Two-way ANOVA followed by post hoc Tukey’s multiple comparisons test. n = 15, 14, 10, and 12 for control RU-, control RU+, RNAi RU-, and RNAi RU+ data respectively. Two-way ANOVA $F_{(1, 47)} = 10.14$, $p = 0.0026$, post hoc Tukey’s multiple comparisons test, *$p < 0.05,$
control RU+ versus RNAi RU+.

n.s.: not significant. Data are mean ± SEM for (B-D) and mean ± SD for (A).

**Figure S3. Expression of gycβ in MB α’β’ neurons** (Related to Figure 3).

(A) Fly brains expressing membrane-tethered GFP (UAS-mCD8::GFP) driven by Gycβ-Gal4<sup>M01568</sup> (green) were colabeled with an anti-FasII antibody (magenta) to mark MB αβ neurons. Weak expression of gycβ in MB αβ neurons was observed. (B) Expression of Gycβ labeled with Gycβ-EGFP in flies carrying the Gycβ[MI08892-GFSTF.2] construct. Gycβ-EGFP signals are shown in the anterior and posterior confocal sections. Gycβ-EGFP signals in MB α’ and β’ neuron in control flies (left panels) were markedly reduced in flies with expression of gycβ<sup>RNAi</sup> in MB α’β’ neurons (right panels). (C) Gycbeta-EGFP signals in the α’ and β’ were significantly reduced in flies expressing gycβ<sup>RNAi</sup> in MB α’β’ neurons using c305a-Gal4 compared with that in the control group. (α lobe, n = 11 and 13 for control and gycβ<sup>RNAi</sup>, <i>t</i>(22) = 0.2234, <i>p</i> = 0.8253; β lobe, n = 9 and 13 for control and gycβ<sup>RNAi</sup>, <i>t</i>(20) = 1.995, <i>p</i> = 0.0599; α’ lobe, n = 11 and 14 for control and gycβ<sup>RNAi</sup>, <i>t</i>(23) = 2.439, *<i>p</i> = 0.0228; β’ lobe, n = 11 and 14 for control and gycβ<sup>RNAi</sup>, <i>t</i>(23) = 10.94, *<i>p</i> < 0.0001, unpaired <i>t</i> test).

Data are mean ± SD.
Figure S4. Expression of gycβ in MB α'β' neurons (Related to Figure 3).

(A) Gycβ-EGFP signals in the α' and β' were significantly reduced in flies expressing gycβRNAi in MB α'[β'] neurons using MB005B-Gal4 compared with that in the control group. (α lobe, n = 4 and 3 for control and gycβRNAi, t(5) = 1.482, p = 0.1984; β lobe, n = 4 and 3 for control and gycβRNAi, t(5) = 0.9719, p = 0.3757; α' lobe, n = 4 and 3 for control and gycβRNAi, t(5) = 2.572, *p = 0.0499; β' lobe, n = 4 and 3 for control and gycβRNAi, t(5) = 7.086, *p = 0.0009, unpaired t test). (B) MB α'β' neuron-specific knockdown of gycβ (MB005B>GycβRNAi) significantly enhanced 3-hr memory compared with that in the control (MB005B>GFP) (3 min, n = 7 for each data, t(12) = 2.178, p = 0.0501; 3 hr, n = 11 and 9 for control and gycβRNAi, t(18) = 2.536, *p = 0.0207, unpaired t test). (C) Shock avoidance test in gycβ knockdown flies. There was no significant difference between the experimental group and the control group in the avoidance of electric shock (n = 7 for each data, U = 24, p = 0.4418, Mann-Whitney U test). (D) Odor avoidance test in gycβ knocked-down flies. There was no significant difference between the experimental group and the control group in the avoidance of odors, Oct and Benz. (Oct, n = 8 and 7 for control and gycβRNAi, t(13) = 0.2384, p = 0.8153; Benz, n = 8 for each data, t(14) = 0.2496, p = 0.8065). (E) MB α'β' neuron-specific knockdown of gycβ (MB463B>GycβRNAi) significantly enhanced 3-min and 3-hr memory compared with control (MB463B>GFP) (3 min, n = 9 and 7 for control and gycβRNAi, t(14) = 7.1, *p < 0.0001; 3 hr, n
= 13 for each data, $t_{(24)} = 3.603$, $*p = 0.0014$, unpaired $t$ test). (F) Shock avoidance test in 
gycβ knockdown flies. There was no significant difference between the experimental group 
and the control group in the avoidance of electric shock ($n = 6$ for each, $t_{(10)} = 0.05725$, $p = 
0.9555$, unpaired $t$ test). (G) Odor avoidance test in gycβ knocked-down flies. There was no 
significant difference between the experimental group and the control group in the avoidance 
of odors, Oct and Benz. (Oct: $n = 8$ for each, $t_{(14)} = 2.693$, $*p = 0.0175$; Benz, $n = 6$ for each 
data, $t_{(10)} = 0.2811$, $p = 0.7844$).

n.s.: not significant. Data are mean ± SEM for all.

**Figure S5. Overexpression of NOS in glia** (Related to Figure 5).

qPCR shows that NOS was overexpressed by 20 times or more by RU feeding in Glia-
GS>NOS flies compared to control flies. (Two-way ANOVA followed by post hoc Tukey’s 
multiple comparisons test. $n = 3$ for each data. Two-way ANOVA $F_{(1, 8)} = 375.4$, $p < 0.0001$. 
post hoc Tukey’s multiple comparisons test, $*p < 0.0001$).

Data are mean ± SD.

**Figure S6. Inhibition of NOS enhances intermediate-term memory in aged flies** 
(Related to Figure 6).
Olfactory memory assay in 20-day-old flies fed L-NAME. L-NAME administration at 100 µM concentration significantly improved 3-hr memory but did not affect 3-min memory (Two-way ANOVA followed by post hoc Tukey’s multiple comparisons test; 3min, n = 7 for each data; 3 hr, n = 11, 12, and 13 for control, 100 µM L-NAME, and 200 µM L-NAME, respectively. Two-way ANOVA $F_{(2, 51)} = 8.134, p = 0.0009$, post hoc Tukey’s multiple comparisons test, *$p=0.0406$, 3 hr-control versus 3 hr-100 µM L-NAME). Data are mean ± SEM.

Quantification of GFP signal in 10-day-old (10d) and 30-day-old (30d) flies expressing mCD8::GFP driven by Gycβ-Gal4. The GFP signals of MB $\alpha$ neurons and $\alpha'$ neurons were increased in aged flies compared with young flies (Two-way ANOVA followed by post hoc Tukey’s multiple comparisons test; n = 9 and 7 for 10d and 30d, respectively. Two-way ANOVA $F_{(1, 28)} = 23.62, p < 0.0001$, post hoc Tukey’s multiple comparisons test, *$p = 0.0393$, MB $\alpha'$ 10 d versus 30 d; *$p = 0.0021$, MB $\alpha$ 10 d versus 30 d; **$p < 0.0001$, MB $\alpha'$ 10 d versus MB $\alpha$ 10 d). Data are mean ± SD.
Figure. S1

A

“Up with age” 827

“KD promotes memory”

4

RNA-seq

RNAi screen

B

“Down with age” 1181

“KD inhibits memory”

54

RNA-seq

RNAi screen

C

3hr memory

Performance Index

RU-

RU+

* *

UAS-\text{cp190 RNAi}

UAS-\text{Gyc6 RNAi}

UAS-amon RNAi

UAS-SA RNAi

Elav-GS
Figure. S3

A

| Gyc β | FasII (α / β) | Merge |
|-------|---------------|-------|
| ![Image] | ![Image] | ![Image] |

B

- **Gyc100B-EGFP[MI08892] + c305a-Gal4 control**
  - anti-GFP (GycB100B)
  - anti-GFP (GycB100B) nc82
- **Gyc100B-EGFP[MI08892] + c305a-Gal4>Gycβ-RNAi**
  - anti-GFP (GycB100B)
  - anti-GFP (GycB100B) nc82

C

- **Relative fluorescence intensity**
  - **Gycβ100B-GFP**
  - **lobes**
  - **α**
  - **β**
  - **α'**
  - **β'**

Legend:
- ○ c305a control
- □ c305a>Gycβ-RNAi

* Significant difference
Figure. S5

Relative Gene Expression

Glia-GS>GFP  Glia-GS>NOS

RU-  RU+

NOS

****
Figure. S6

A

Aged (20d)

Performance Index

3 min 3 hr

control

100µM L-NAME

200µM L-NAME

B

Gyc>mCD8::GFP

GFP (a.u.)

MBα' MBα

10d 30d
Table S1. List of genes that are negative-memory regulators with age-dependent upregulating gene expression

| Gene ID      | CG number | Gene Name    | BaseMean  | log2FoldChange | pvalue   |
|--------------|-----------|--------------|-----------|----------------|----------|
| FBgn0000283  | CG6384    | Cp190        | 4086.674094 | 0.345788762    | 4.86E-06 |
| FBgn0013973  | CG1470    | Gycbeta100B  | 5417.554561 | 0.248444822    | 2.10E-07 |
| FBgn0020616  | CG3423    | SA           | 1523.413465 | 0.220247667    | 0.001137266 |
| FBgn0023179  | CG6438    | amon         | 13543.6522  | 0.277229669    | 5.25E-08 |
Table S2. List of genes that are positive-memory regulators with age-dependent downregulating gene expression

| Gene ID     | CG number | Gene Name | BaseMean | log2FoldChange | pvalue     |
|-------------|-----------|-----------|----------|----------------|------------|
| FBgn0000422 | CG10697   | Ddc       | 10209.29924 | -0.161249      | 0.009236328 |
| FBgn0003423 | CG1417    | slgA      | 13788.04981 | -0.212593      | 4.45E-06   |
| FBgn0003475 | CG10076   | spir      | 7847.563597 | -0.18385       | 1.68E-04   |
| FBgn0004623 | CG8770    | Gbeta76C  | 17878.20036 | -0.370805      | 2.09E-11   |
| FBgn0004903 | CG6354    | Rb97D     | 2092.141754 | -0.292246      | 9.59E-06   |
| FBgn0005561 | CG11049   | sv        | 972.4737607 | -0.72329       | 8.20E-10   |
| FBgn0006075 | CG16858   | vkg       | 1642.218135 | -0.363091      | 1.67E-07   |
| FBgn0016075 | CG3234    | tim       | 37545.90429 | -0.349188      | 6.18E-11   |
| FBgn0016075 | CG15771   | CG15771   | 3226.250201 | -0.221155      | 2.47E-05   |
| FBgn0021979 | CG3082    | (2)k09913 | 6423.14735 | -0.273099      | 6.30E-06   |
| FBgn0024963 | CG7535    | GluClalpha| 16440.20957 | -0.164068      | 0.004641559 |
| FBgn0025549 | CG1659    | unc-119   | 2733.079954 | -0.186491      | 0.00488067 |
| FBgn0029819 | CG3016    | Usp30     | 1284.807535 | -0.211158      | 0.005081925 |
| FBgn0029830 | CG14447   | Grip      | 249.8349895 | -0.363879      | 0.00510487 |
| FBgn0030087 | CG7766    | CG7766    | 6517.94836  | -0.273341      | 1.01E-07   |
| FBgn0030668 | CG8128    | CG8128    | 500.6648223 | -0.341756      | 4.91E-04   |
| FBgn0030670 | CG9245    | Pis       | 9257.448139 | -0.315721      | 6.65E-07   |
| FBgn0030895 | CG7135    | CG7135    | 2082.340104 | -0.351498      | 1.21E-04   |
| FBgn0031998 | CG8451    | SLC5A11   | 700.5792629 | -0.618638      | 1.47E-08   |
| FBgn0032021 | CG7781    | CG7781    | 7826.390416 | -0.231729      | 2.72E-06   |
| FBgn0032729 | CG10639   | L2HGDH    | 547.6830392 | -0.339418      | 9.66E-04   |
| FBgn0033434 | CG1902    | CG1902    | 1446.078606 | -0.187497      | 0.004702246 |
| FBgn0034051 | CG8295    | Milf      | 6958.798379 | -0.237691      | 5.05E-04   |
| FBgn0034576 | CG9350    | ND-B14.7  | 2431.818654 | -0.29144       | 0.004488762 |
| FBgn0034902 | CG5532    | CG5532    | 285.6281657 | -0.388102      | 0.003379101 |
| FBgn0035526 | CG1316    | CG1316    | 4604.477238 | -0.216033      | 1.05E-05   |
| FBgn0035695 | CG10226   | CG10226   | 2060.916751 | -0.373209      | 9.76E-06   |
| FBgn0036043 | CG8177    | Ae2       | 13322.09774 | -0.446129      | 2.84E-15   |
| FBgn0036428 | CG9238    | Gbs-70E   | 4244.302115 | -0.732464      | 3.84E-11   |
| FBgn0037138 | CG7145    | P5CDh1    | 13591.10786 | -0.174893      | 2.94E-04   |
| FBgn0037607 | CG8036    | CG8036    | 3863.597691 | -0.467017      | 3.35E-05   |
| GeneID     | Cluster | GeneName | RankScore | RnkScore | SigScore |
|-----------|---------|----------|-----------|----------|----------|
| FBgn0037655 | CG11984 | Kcmf1    | 5879.36327 | -0.138537 | 0.005413205 |
| FBgn0038610 | CG7675  | CG7675   | 960.7764541 | -0.444555 | 2.18E-06 |
| FBgn0039132 | CG5864  | AP-1sigma| 2364.048263 | -0.176463 | 0.002752215 |
| FBgn0039635 | CG11876 | Pdhb     | 3143.669328 | -0.617765 | 1.41E-14 |
| FBgn0039748 | CG15529 | CG15529  | 333.3041799 | -0.519183 | 4.12E-06 |
| FBgn0051005 | CG31005 | qless    | 772.5825998 | -0.28746 | 0.001408794 |
| FBgn0051352 | CG31352 | Unc-115a | 2230.638195 | -0.177975 | 0.0042323 |
| FBgn0052000 | CG32000 | anne     | 22296.54772 | -0.199862 | 0.00689574 |
| FBgn0052672 | CG32672 | Atg8a    | 11275.86514 | -0.249744 | 4.32E-06 |
| FBgn0054974 | CG34376 | CG34376  | 1344.174565 | -0.477632 | 7.53E-12 |
| FBgn005673 | CG13272 | CG13272  | 42.24566195 | -1.278612 | 3.91E-10 |
| FBgn0259111 | CG42253 | Ndae1    | 4515.954776 | -0.16187 | 0.003318863 |
| FBgn0260743 | CG18347 | GC1      | 1836.300869 | -0.244418 | 5.70E-05 |
| FBgn0261477 | CG5186  | slim     | 2604.24922 | -0.19727 | 6.26E-04 |
| FBgn0261955 | CG3861  | kdn      | 12888.21548 | -0.572736 | 4.44E-31 |
| FBgn0262476 | CG43066 | CG43066  | 9067.295112 | -0.383012 | 1.19E-12 |
| FBgn0263199 | CG5288  | Galk     | 1208.850134 | -0.647784 | 4.61E-07 |
| FBgn0263776 | CG43693 | CG43693  | 1318.304619 | -0.471205 | 2.65E-08 |
| FBgn0264308 | CG43778 | CG43778  | 8208.064806 | -0.314511 | 5.36E-05 |