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# Dietary methionine supplementation improves cognitive dysfunction associated with transsulfuration pathway upregulation in subacute aging mice

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To explore the effects of methionine (Met) supplementation on cognitive dysfunction and the associated mechanisms in aging mice. The mice were administrated 0.15 g/kg/day D-galactose subcutaneously and fed a normal (0.86% Met) or a Met-supplemented diet (1.72% Met) for 11 weeks. Behavioral experiments were conducted, and we measured the plasma metabolite levels, hippocampal and plasma redox and inflammatory states, and hippocampal transsulfuration pathway-related parameters. Met supplementation prevented aging-induced anxiety and cognitive deficiencies, and normalized the plasma levels of multiple systemic metabolites (e.g., betaine, taurine, and choline). Furthermore, dietary Met supplementation abolished oxidative stress and inflammation, selectively modulated the expression of multiple cognition-related genes and proteins, and increased flux via the transsulfuration pathway in the hippocampi of aging mice, with significant increase in H<sub>2</sub>S and glutathione production. Our findings suggest that dietary Met supplementation prevented cognitive deficiencies in aging mice, probably because of increased flux via the transsulfuration pathway.

Increasing human lifespan leads to an increase in debilitating conditions, such as cognitive decline. Cognitive decline is a common feature of the aging nervous system and is a key risk factor for neurodegenerative diseases including Alzheimer's disease (AD), which is characterized by progressive memory loss and a decline in mental capacity<sup>1</sup>. Oxidative stress and inflammation interact with each other, and this interaction contributes to the characteristics of aging and the essential components of related pathological pathways, thus driving age-related cognitive decline, including the development of AD<sup>2,3</sup>. Excess reactive oxygen species (ROS) accompanied with aging cause damage to lipid peroxidation, protein oxidation, nuclear DNA oxidation, and mitochondrial dysfunction in the brain, eventually causing neuronal metabolism disorder and neuron damage<sup>3</sup>. The damage such as DNA damage further induces inflammation through activation of nuclear factor kappa B (NF- $\kappa$ B)<sup>4</sup>, which degrades the ability of neuron to resist risks and repair cellular damage, exacerbating the occurrence of oxidative stress and creating a vicious circle, eventually causing age-related cognitive decline<sup>5</sup>. Reducing oxidative stress and inflammation in the brain through antioxidant supplementation presents an effective strategy for treating aging-associated cognitive impairment<sup>6,7</sup>. The transsulfuration

pathway (TSP) serves as a central hub for the metabolism of sulfur-containing amino acids<sup>8</sup>, playing critical roles in redox homeostasis and stress response integration<sup>9</sup>. The TSP generates several sulfur metabolites, including hydrogen sulfide (H<sub>2</sub>S), glutathione (GSH), and taurine. H<sub>2</sub>S, GSH, and taurine are among the most important antioxidants that regulate the redox and inflammatory states in the brain, and they also directly modulate neuronal signaling pathways to regulate learning and memory function. Aging is usually accompanied by dysregulation of the TSP, and increased flux via the TSP is a key target to modulate cognitive function, slow the aging process, and increase lifespan<sup>8,10</sup>.

Methionine (Met), the only sulfur-containing amino acid among the essential amino acids, is the initial amino acid used for protein synthesis. In particular, Met acts as the core and initiating substrate of the TSP in mammals. In the TSP, dietary Met is converted to homocysteine, which is further converted to cystathionine by cystathionine- $\beta$ -synthase (CBS), and cystathionine is further acted on by cystathionine- $\gamma$ -lyase (CSE) to generate cysteine. Finally, cysteine is converted to essential antioxidants such as GSH and H<sub>2</sub>S and other non-essential amino acid such as taurine<sup>9</sup>. Met-supplemented rice proteins (additional supplementation with 0.5, 1.0, and

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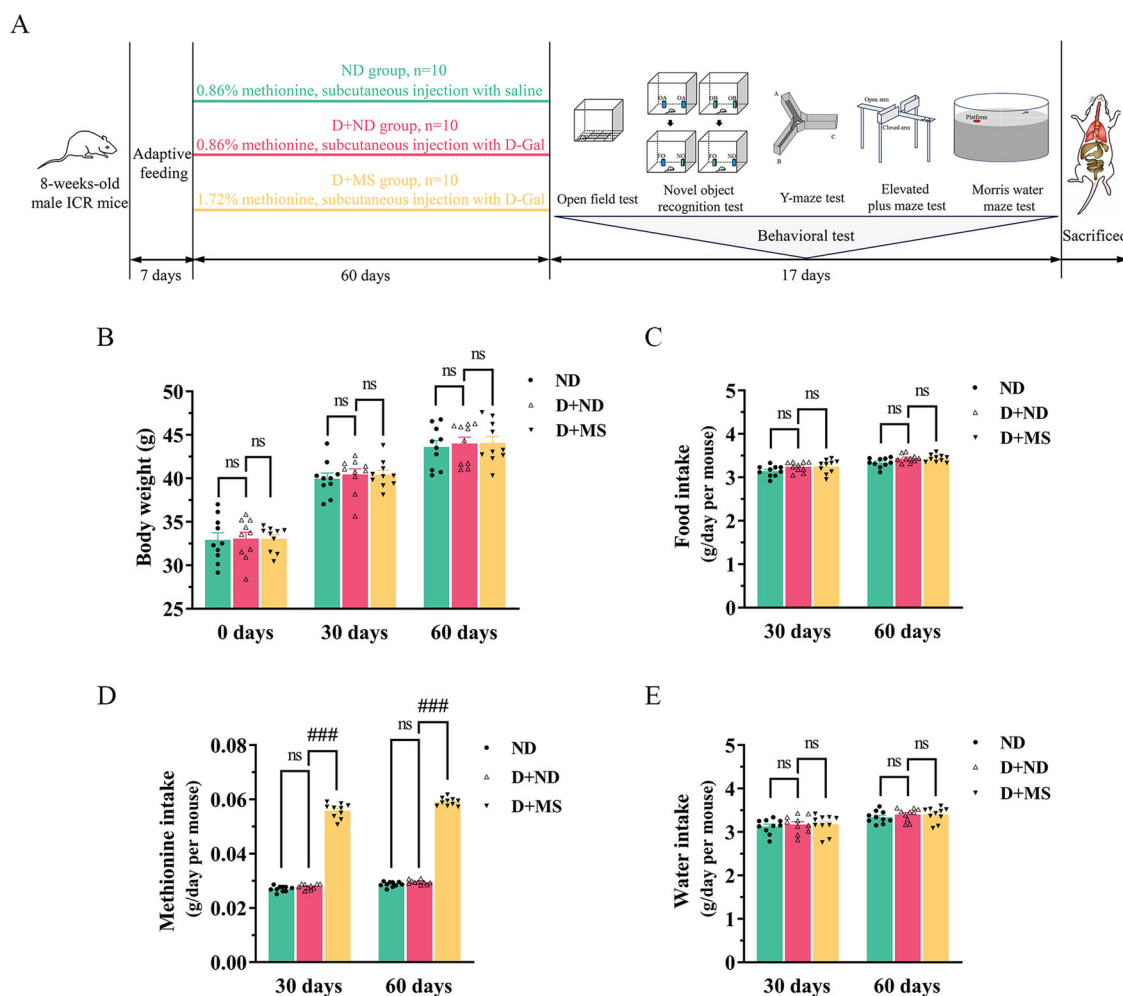
1.5-fold of Met) can enhance GSH synthesis to augment endogenous antioxidant activity in rats<sup>11</sup>. Dietary Met supplementation with 1.5% and 1.3% Met can reduce oxidative stress and inflammation induced by an atherogenic diet and a high-fat fructose diet, respectively, in rats<sup>12,13</sup>. Moreover, appropriate dietary supplementation with Met (e.g., additional supplementation with 0.5% Met in the diet or oral administration of 0.1–0.25 g/kg body weight of Met) improved hepatic steatosis, insulin resistance, fibrosis, and bone health in several animal experimental models<sup>14–16</sup> and reduced pain in patients with chronic pancreatitis<sup>17</sup>. Stimulating the TSP may be responsible for these aforementioned effects, potentially reducing oxidative stress by enhancing the availability of antioxidants, including H<sub>2</sub>S, GSH, taurine, and cysteine. Furthermore, based on the important role of Met metabolism in the brain, appropriate dietary supplementation with Met has many benefits for the central nervous system (CNS). In vitro, 0.5 or 1.0 mM L-Met significantly protects against rat brain synaptosomes from oxidative stress induced by low concentrations of tert-butylhydroperoxide<sup>18</sup>. In vivo, early supplementation with Met combined with B vitamins can prevent early stress-induced cognitive impairment<sup>19</sup>. The total Met intake was positively correlated with cognition in the data of 4852 Chinese adults aged  $\geq 55$  years<sup>20</sup>, and the intake of high-quality protein sources was found to prevent cognitive deficits in older individuals<sup>21,22</sup>. However, whether appropriate dietary supplementation with Met can ameliorate aging-induced cognitive decline and whether the underlying mechanism is related to the alterations in the TSP remain unclear.

In animal models of aging, D-Galactose (D-Gal) induced aging model is highly favored because of its convenience, least side effects, and the higher survival rate<sup>23</sup>. Excess D-Gal can be metabolized to produce aldoses and hydrogen peroxide to stimulate ROS production to construct a model very similar to normal aging. In this study, a D-Gal-induced subacute aging model was used to assess the effects of Met supplementation on cognitive dysfunction and the associated mechanisms. Various behavioral experiments were performed to determine these effects. Furthermore, metabolite levels in the plasma were analyzed, and the redox and inflammatory states in the hippocampi and plasma were assessed. In particular, TSP-related metabolism in the hippocampi was investigated in detail.

## Results

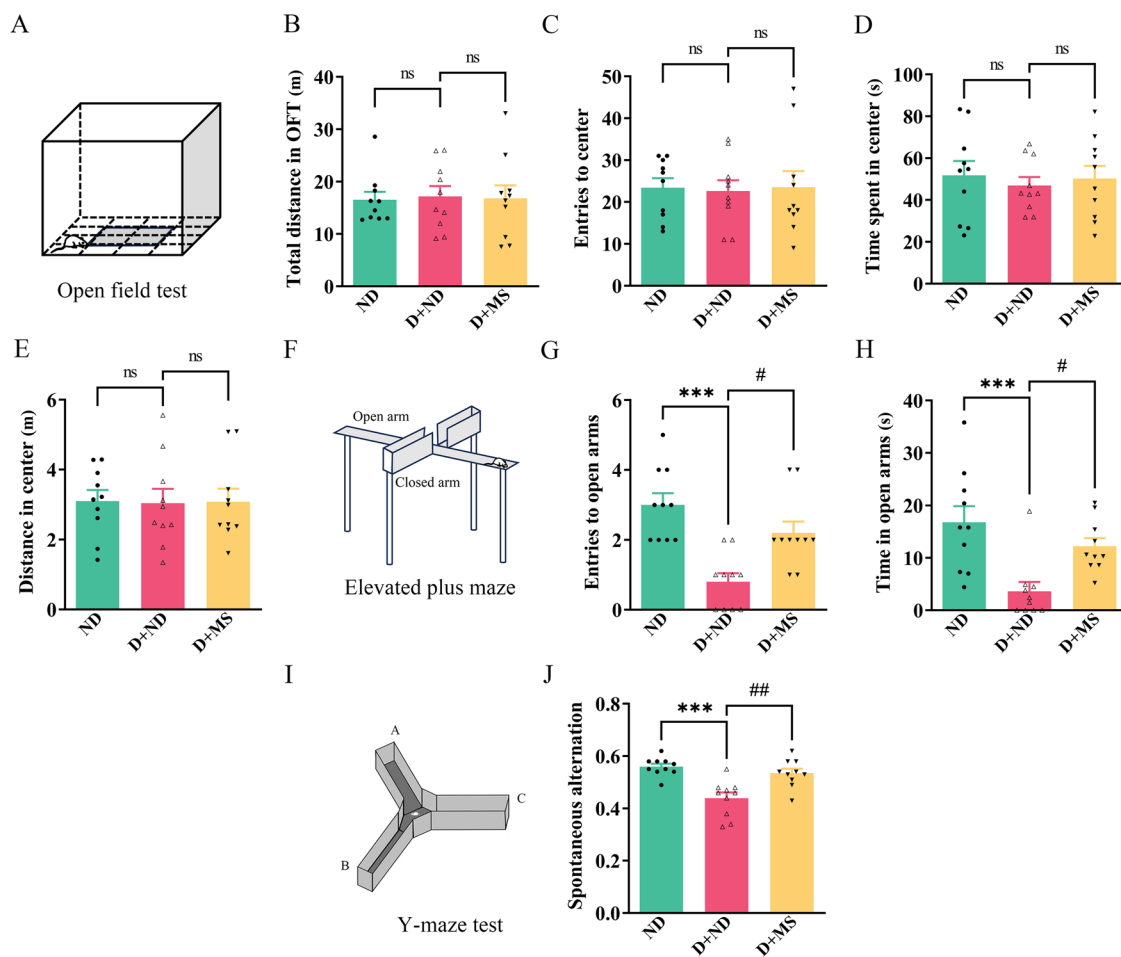
### Effects of Met supplementation on body weight, food intake, Met intake, and water intake in subacute aging mice

On day 0, the weights of mice in all groups were similar. After 60 days of feeding, no significant differences in body weight were observed between the groups (Fig. 1B). On measuring the food intake of each group after feeding for 30 and 60 days, no significant differences were observed between the groups (Fig. 1C). Met intake was calculated based on the food intake after 30 and 60 days of feeding and was significantly higher in the D + MS group than in the D + ND group (Fig. 1D). Furthermore, no significant differences were observed with respect to the measured water intake between the groups (Fig. 1E).



**Fig. 1 | Effects of Met supplementation on body weight, food intake, Met intake, and water intake in subacute aging mice. A** study diagram, **B** body weight, **C** food intake, **D** Met intake, **E** water intake. ND: saline + normal diet; D + ND: D-Gal +

normal diet; D + MS: D-Gal + Met supplementation. Data are represented as mean  $\pm$  SEM ( $n = 10$ ). “ns” means not significantly different; “###”  $p < 0.001$ , versus D + ND.



**Fig. 2 | Effects of Met supplementation on anxiety and working memory in subacute aging mice.** **A** OFT diagram (anxious mice will move less into the central area and more along the wall), **B** total distance in the OFT, **C** entries to the central area in the OFT, **D** time spent in the central area in the OFT, **E** distance in the central area in the OFT, **F** EPMT diagram (anxiety mice will less enter the open arms),

**G** entries to the open arms in the EPMT, **H** time spent in the open arms in the EPMT, **I** Y-maze test diagram, **J** spontaneous alternation. ND: saline + normal diet; D + ND: D-Gal + normal diet; D + MS: D-Gal + Met supplementation. Data are represented as mean  $\pm$  SEM ( $n = 10$ ). “ns” means not significantly different; \*\*\* $p < 0.001$ , versus ND; # $p < 0.05$ , ## $p < 0.01$ , versus D + ND.

### Effects of Met supplementation on anxiety and working memory in subacute aging mice

No differences were observed between the open-field test (OFT) results of the different groups (Fig. 2A–E). In the elevated plus maze test (EPMT) (Fig. 2F), the total distance covered did not differ significantly between the groups (Supplementary File, Fig. S1). The number of entries into the open arms and the time spent in the open arms of the maze were significantly decreased in the D + ND group compared to that in the ND group, whereas they were significantly increased in the D + MS group compared to that in the D + ND group (Fig. 2G, H). In the Y-maze test (YMT) (Fig. 2I), spontaneous alternation rates were significantly decreased in the D + ND group compared to that in the ND group, whereas it was significantly increased in the D + MS group compared to that in the D + ND group (Fig. 2J).

### Effects of Met supplementation on non-spatial recognition memory and spatial memory in subacute aging mice

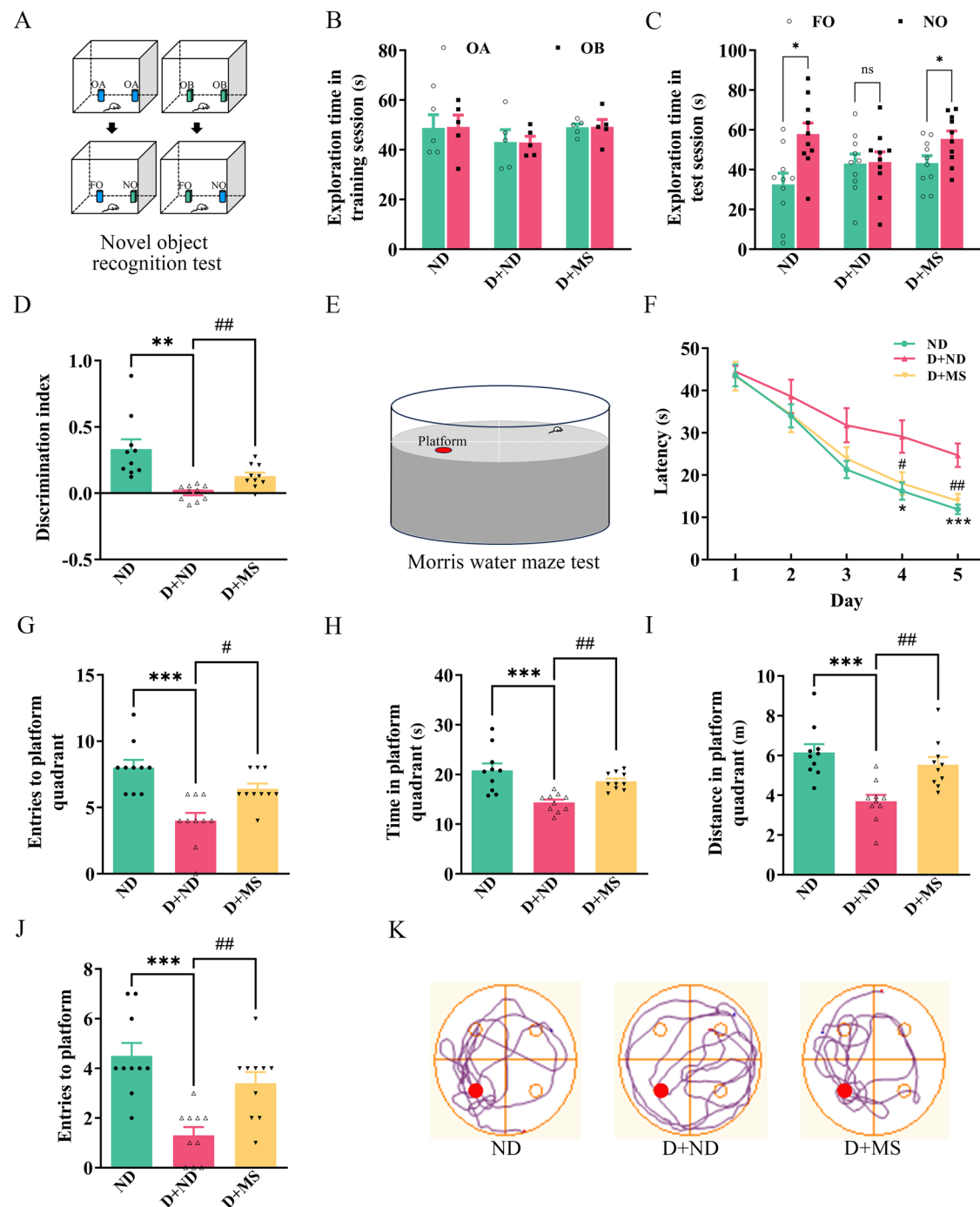
In the novel object recognition test (NORT) (Fig. 3A), when the box contained two identical objects, the mice spent the same time exploring both objects (Fig. 3B). When an object was replaced by a new object at the same location, the time spent investigating the new object was significantly longer than the time that spent investigating the old object in the ND and D + MS groups ( $p < 0.05$ , Fig. 3C), whereas the D + ND group showed no significant difference ( $p > 0.05$ , Fig. 3C). The discrimination index (DI) value was significantly decreased in the D + ND group compared to that in the ND

group, whereas it was significantly increased in the D + MS group compared to that in the D + ND group (Fig. 3D).

In the Morris water maze test (MWMT) (Fig. 3E), no differences in latency or swimming speed were observed between the groups when the platform was out of the water (Supplementary Fig. S1). All experimental groups showed a progressive decrease in latency when the platform was below the water surface (Fig. 3F). On day 5, dietary Met supplementation reduced the escape latency by 43.7% compared to that in the D + ND group (Fig. 3F). When the platform was removed on the last day, several indicators related to activities in the target quadrant were significantly decreased in the D + ND group compared to those in the ND group, whereas they were significantly increased in the D + MS group compared to those in the D + ND group (Fig. 3G–J). Furthermore, mice in the ND and D + MS groups were more inclined to swim in the target quadrant than the mice in the D + ND group (Fig. 3K).

### Effects of Met supplementation on plasma metabolite levels in subacute aging mice

The plasma metabolic profiles were analyzed using nuclear magnetic resonance (NMR) spectroscopy. Three representative spectra randomly selected from each group are shown in Fig. 4A. Fifty metabolites were unambiguously identified based on the spectral signals. Detailed information on these metabolites is provided in Supplementary Table S3. A principal component analysis (PCA) score plot (Fig. 4B) showed that no outliers were present in the



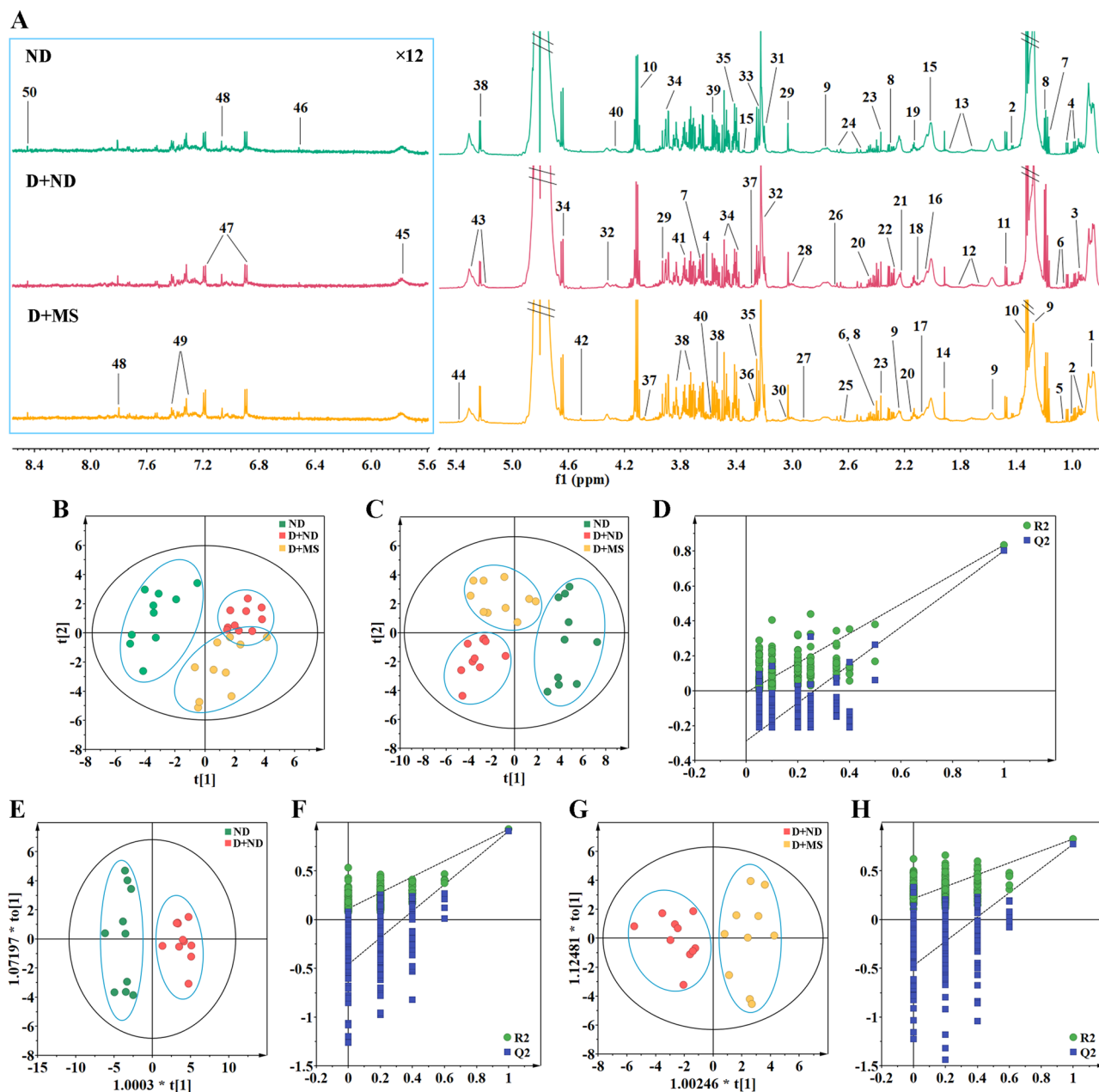
**Fig. 3 | Effects of Met supplementation on non-spatial recognition memory and spatial memory in subacute aging mice.** **A** NORT diagram (mice with better non-spatial declarative memory will explore new objects longer), **B** exploration time for two same objects (OA means object A, OB means object B), **C** exploration time for two different objects (FO means familiar object, NO means novel object), **D** discrimination index, **E** MWM diagram (mice with better spatial memory show less latency and more activity in the target quadrant), **F** latency during daily training

trials, **G** entries to the platform quadrant, **H** time spent in the platform quadrant, **I** distance travelled in the platform quadrant, **J** entries to the platform, **K** swimming track diagram. ND: saline + normal diet; D + ND: D-Gal + normal diet; D + MS: D-Gal + Met supplementation. Data are represented as mean  $\pm$  SEM ( $n = 10$ ). “ns” means not significantly different; \* $p < 0.05$ , \*\* $p < 0.05$ , \*\*\* $p < 0.001$ , versus ND; # $p < 0.05$ , ## $p < 0.01$ , versus D + ND.

dataset and that the distances between the groups did not differ significantly. However, in the partial least-squares discriminant analysis (PLS-DA; Fig. 4C) and orthogonal PLS-DA (OPLS-DA; Fig. 4E, G) score plots, the distances between groups differed significantly. Nevertheless, all  $R^2$  and  $Q^2$  values were greater than 0.5 in all the three models, suggesting their suitability and predictability. Furthermore, permutation tests (Fig. 4D, F, H) indicated that the PLS-DA and OPLS-DA models were robust and did not overfit the data.

Finally, based on the variable importance in the projection (VIP) values and  $p$ -values ( $VIP > 1$  and  $p < 0.05$ ), potential metabolic biomarkers were identified and are presented in Fig. 5.

Plasma betaine, creatine, myo-inositol, glutamine, acetate, propionate, fumarate, taurine, Met, tyrosine, leucine, glycine, phenylalanine, alanine, and formate levels were significantly decreased, whereas pyruvate, allantoin, N-acetyl glycoprotein, O-acetyl glycoprotein, urea,



**Fig. 4 | Representative 600 MHz  $^1\text{H}$ -NMR spectra of plasma samples.** A PCA score plots (B:  $R^2X = 0.641$ ,  $Q^2 = 0.522$ ), PLS-DA score plots (C:  $R^2X = 0.843$ ,  $R^2Y = 0.757$ ,  $Q^2 = 0.608$ ), PLS-DA validation plots (D, permutation number: 200), OPLS-DA score plots (E: D + ND vs ND,  $R^2X = 0.885$ ,  $R^2Y = 0.929$ ,  $Q^2 = 0.909$ ; G: D + MS vs D + ND,  $R^2X = 0.802$ ,  $R^2Y = 0.828$ ,  $Q^2 = 0.775$ ), and OPLS-DA validation plots (F:

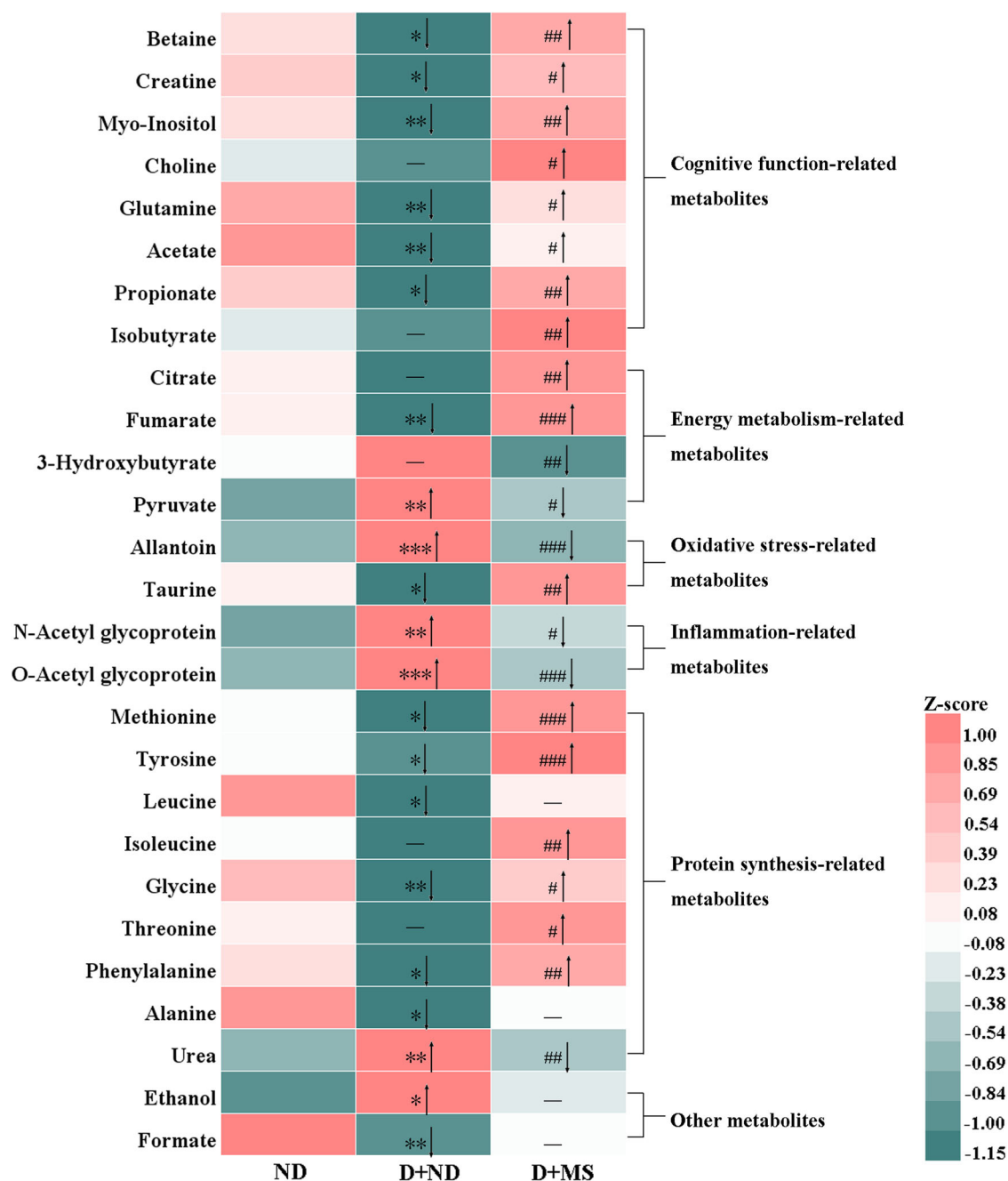
D + ND vs ND, permutation number: 200; H: D + MS vs D + ND, permutation number: 200) according to the  $^1\text{H}$ -NMR spectra of metabolites in the plasma obtained from the ND (green circles), HFD (red circles), and MRD (yellow circles) group mice ( $n = 10$ ). ND: saline + normal diet; D + ND: D-Gal + normal diet; D + MS: D-Gal + Met supplementation.

and ethanol levels were significantly increased in the D + ND group compared to those in the ND group (Fig. 5). In contrast, the levels of betaine, creatine, myo-inositol, choline, glutamine, acetate, propionate, isobutyrate, citrate, fumarate, taurine, Met, tyrosine, isoleucine, glycine, threonine, and phenylalanine were significantly increased in the D + MS group compared to those in the D + ND group, and 3-hydroxybutyrate, pyruvate, allantoin, N-acetyl glycoprotein, O-acetyl glycoprotein, and urea levels were significantly decreased in the D + MS group compared to those in the D + ND group (Fig. 5,  $p < 0.05$ ).

#### Effects of Met supplementation on the redox state in the hippocampus and plasma in subacute aging mice

In the blood and plasma, the levels of ROS and malondialdehyde (MDA) produced by ROS stimulation were significantly increased in the D + ND

group compared to those in the ND group, whereas they were significantly increased in the D + MS group compared to those in the D + ND group (Fig. 6A, B). The levels of oxidation resistance indicators (total superoxide dismutase (T-SOD) and total antioxidant capacity (T-AOC)) were significantly decreased in the D + ND group compared to those in the ND group, whereas they were significantly increased in the D + MS group compared to those in the D + ND group (Fig. 6C, D). In the brain, the levels of ROS and harmful products produced by ROS stimulation (MDA and advanced oxidation protein products (AOPPs)) were significantly increased in the D + ND group compared to those in the ND group, whereas they were significantly decreased in the D + MS group compared to those in the D + ND group (Fig. 6E–G). The oxidation resistance indicator reduced glutathione/oxidized glutathione (GSH/GSSG) was significantly decreased in the D + ND group compared to that in the ND group, whereas it was



**Fig. 5 | Potential metabolic biomarkers in the plasma.** The heat map exhibits normalized relative metabolite levels by the equation  $Z\text{-score} = (\text{value in each sample} - \text{average value in all samples}) / (\text{standard deviation of value in all samples})$ . “↑/↓” indicates significantly increased/decreased, “—” indicates insignificant. ND: saline +

normal diet; D + ND: D-Gal + normal diet; D + MS: D-Gal + Met supplementation. Data are represented as mean  $\pm$  SEM ( $n = 10$  mice). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , versus ND; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , versus D + ND.

significantly increased in the D + MS group compared to that in the D + ND group (Fig. 6H). The levels of the other two oxidation resistance indicators (T-SOD and T-AOC) did not differ significantly between the groups (Fig. 6I, J).

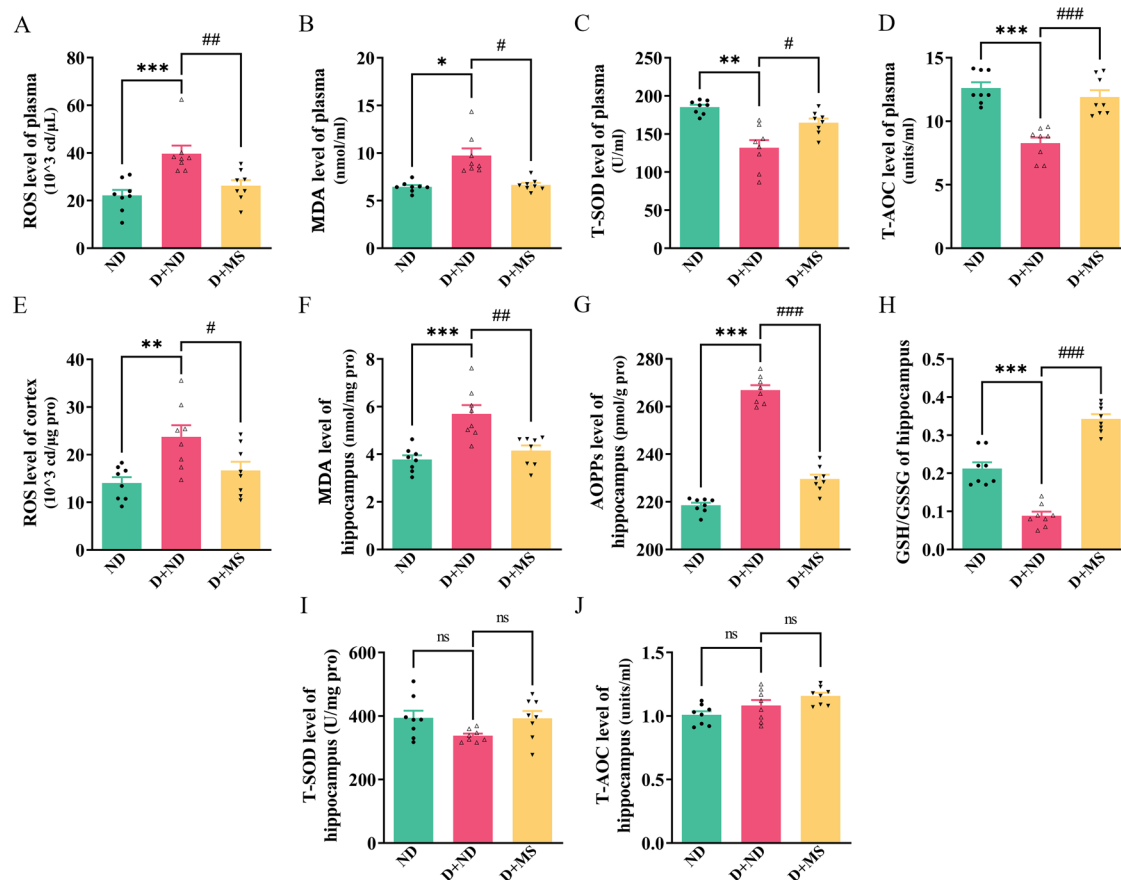
#### Effects of Met supplementation on the inflammatory state in the hippocampi and plasma in subacute aging mice

Plasma levels of interleukin 1 beta (IL-1 $\beta$ ), IL-6, and tumor necrosis factor alpha (TNF- $\alpha$ ) were significantly increased in the D + ND group compared to those in the ND group, whereas they were significantly decreased in the D + MS group compared to those in the D + ND group (Fig. 7A–C). However, IL-10 levels showed an opposite trend (Fig. 7D). In the hippocampi,

there was no significant difference in IL-1 $\beta$  levels between the groups (Fig. 7E). IL-6 and TNF- $\alpha$  levels were significantly increased in the D + ND group compared to those in the ND group, whereas they were significantly decreased in the D + MS group compared to those in the D + ND group (Fig. 7F, G). Conversely, IL-10 levels exhibited an opposite trend (Fig. 7H).

#### Effects of Met supplementation on the accumulation of cognitive toxins and cognition-associated pathways in the hippocampi in subacute aging mice

Furthermore, the accumulation of cognitive toxins and the expression of cognition-related genes in the hippocampi were determined in subacute



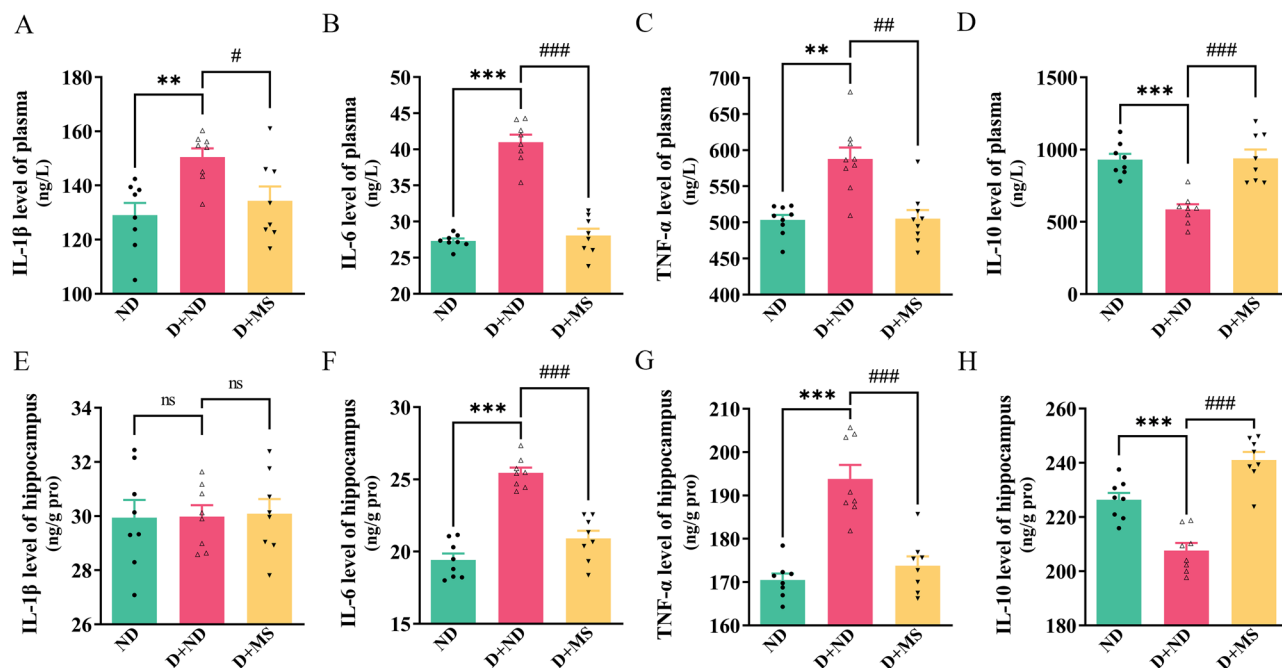
**Fig. 6 | Effects of Met supplementation on the redox state in the blood and hippocampi in subacute aging mice.** A ROS level in the blood, B MDA, C T-SOD, and D T-AOC levels in the plasma, E ROS level in cortex, F MDA, G AOPPs, H GSH/GSSG, I T-SOD, and J T-AOC levels in the hippocampi. ND: saline + normal diet;

D + ND: D-Gal + normal diet; D + MS: D-Gal + Met supplementation. Data are represented as mean  $\pm$  SEM ( $n = 8$ ). “ns” means not significantly different; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , versus ND; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ , versus D + ND.

aging mice. The levels of aging-related toxins, including advanced glycation end products (AGEs), lipofuscin, and amyloid beta peptide 40 (A $\beta$ -40), were significantly increased in the D + ND group compared to those in the ND group, whereas they were significantly decreased in the D + MS group compared to those in the D + ND group (Fig. 8A–C). The relative mRNA expression levels of NF- $\kappa$ B, nuclear factor erythroid-derived 2-like 2 (Nrf2), cysteinyl aspartate-specific proteinase 3 (Caspase3), and Bcl-2 associated x protein (Bax) were decreased in the D + MS group compared to those in the D + ND group, whereas the expression levels of heme oxygenase 1 (HO-1) and NADPH quinone oxidoreductase-1 (NQO-1) were significantly increased (Fig. 8D). In addition, the relative mRNA expression levels of brain-derived neurotrophic factor (BDNF), tyrosine kinase receptor B (TrkB), neurogranin (RC3), neuromodulin (Gap-43), postsynaptic density protein 95 (PSD-95), synaptopodin (SYNPO), N-methyl-D-aspartate receptor 2b (Nr2b), and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CAMK2) alpha chain and beta chain (CaMK2B and CaMK2D) were significantly decreased in the D + ND group compared to those in the ND group, whereas they were significantly increased in the D + MS group compared to those in the D + ND group (Fig. 8E, F). Furthermore, western blotting showed that P-NF- $\kappa$ B p65/NF- $\kappa$ B p65 in the hippocampi was significantly increased in the D + ND group compared to that in the ND group, whereas it was significantly decreased in the D + MS group compared to that in the D + ND group (Fig. 8G, H). BDNF, Nr2b, and P-CAMK2/CAMK2 levels in the hippocampi were decreased by 74.4%, 46.7%, and 52.4% in the D + ND group compared to those in the ND group, respectively, whereas they were increased by 64.9%, 225.4%, and 56.3% in the D + MS group compared to that in the D + ND group, respectively (Fig. 8G, H).

### Effects of Met supplementation on the transsulfuration pathway in the hippocampi in subacute aging mice

The effects of Met supplementation on the TSP in the hippocampi of subacute aging mice were determined. Met levels in the plasma and hippocampi were significantly decreased in the D + ND group compared to those in the ND group, whereas they were significantly increased in the D + MS group compared to those in the D + ND group (Fig. 9A, B). S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels in the hippocampi showed the same trend as that shown by the Met levels (Fig. 9C, D), and the SAM/SAH ratio was significantly decreased in the D + MS group compared to that in the D + ND group. Furthermore, the relative mRNA expression levels of methylenetetrahydrofolate reductase (MTHFR), betaine homocysteine methyltransferase (BHMT), and glycine N-methyltransferase (GNMT) were significantly decreased in the D + ND group compared to those in the ND group, whereas they were significantly increased in the D + MS group compared to those in the D + ND group. However, there was an opposite trend in methionine synthase (MS) expression levels (Fig. 9F). Hcy levels in the plasma and hippocampi were significantly increased in the D + ND group compared to those in the ND group, whereas no significant difference was observed between the D + MS and D + ND groups (Fig. 9G, H). In addition, cysteine, H<sub>2</sub>S, and GSH levels and the expression levels of the key enzymes for H<sub>2</sub>S and GSH production, including CBS, CSE, and glutathione synthetase (GS), in the hippocampi, were significantly decreased in the D + ND group compared to those in the ND group, whereas they were significantly increased in the D + MS group compared to those in the D + ND group (Fig. 9I–N). Western blotting showed that CBS level in the hippocampi was decreased by 63.2% in the D + ND group compared to that in the ND group, whereas it was increased



**Fig. 7 | Effects of Met supplementation on the inflammatory state in the blood and hippocampi in subacute aging mice.** A IL-1 $\beta$ , B IL-6, C TNF- $\alpha$ , and D IL-10 levels in the plasma, E IL-1 $\beta$ , F IL-6, G TNF- $\alpha$ , and H IL-10 levels in the hippocampi. ND: saline + normal diet; D + ND: D-Gal + normal diet; D + MS: D-Gal + Met

supplementation. Data are represented as mean  $\pm$  SEM ( $n = 8$  mice). “ns” means not significantly different; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , versus ND; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ , versus D + ND.

by 152.4% in the D + MS group compared to that in the D + ND group (Fig. 9O, P). A pathway diagram indicating the relationships between Met metabolism and H<sub>2</sub>S and GSH production in the hippocampi is shown in Fig. 10A. The correlations between the levels of H<sub>2</sub>S and GSH in the hippocampi, behavioral index value, and other important biochemical indicators levels were further confirmed (Fig. 10B). H<sub>2</sub>S and GSH levels correlated positively with cognitive improvement, and they correlated negatively with oxidative stress and inflammation.

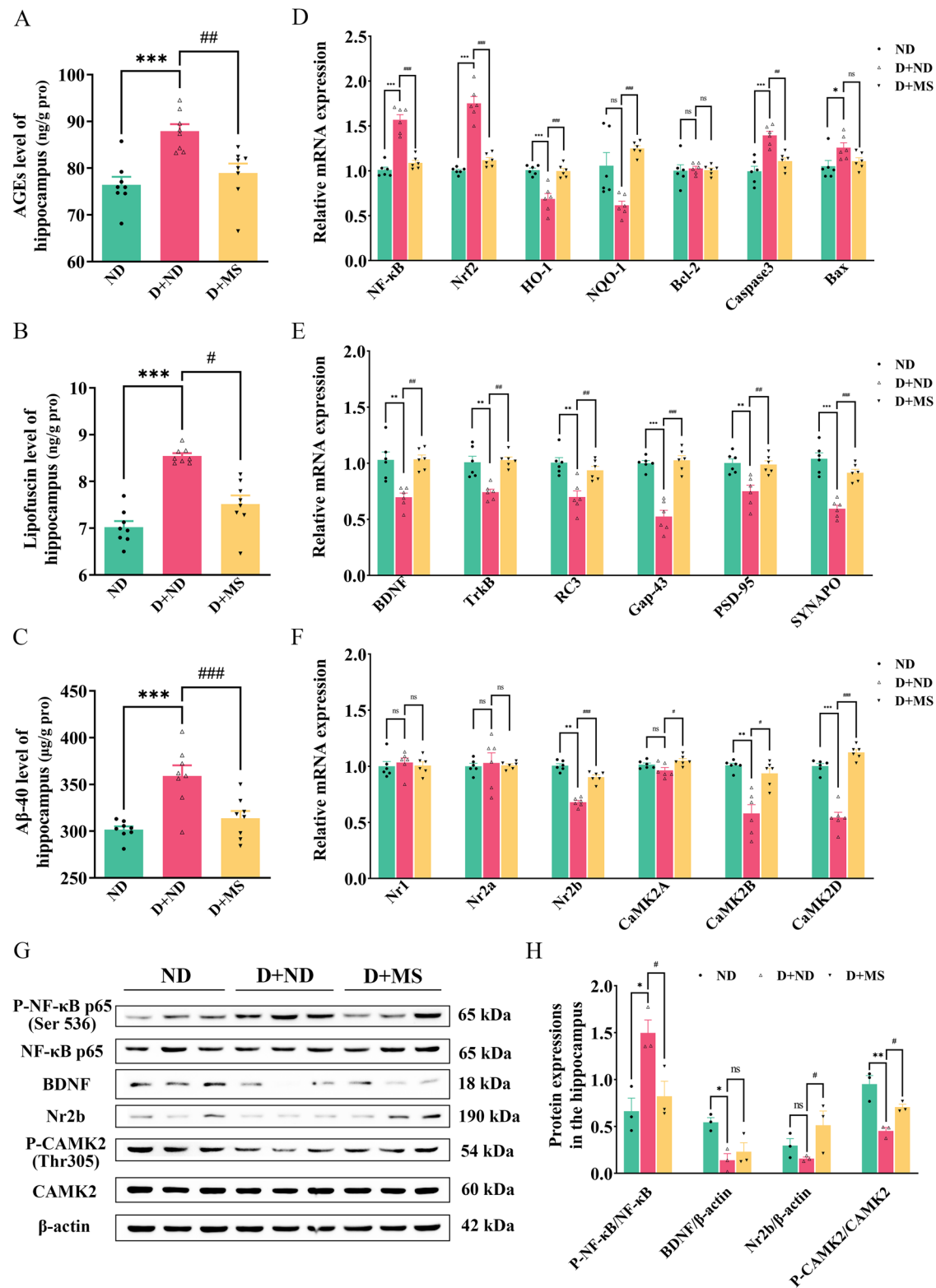
## Discussion

Here, to first demonstrate the effects of dietary Met supplementation on cognitive function in subacute aging mice, several behavioral experiments were conducted. Furthermore, plasma metabolite levels, redox and inflammatory states, and H<sub>2</sub>S- and GSH-generating pathways in the hippocampi were evaluated. Our findings indicate that appropriate dietary Met supplementation increased flux via the TSP, which may improve cognitive impairment in subacute aging mice.

Dietary Met supplementation improves cognitive dysfunction in subacute aging mice. Clinically, older patients with dementia frequently show a cluster of behavioral disturbances, including learning disorders, amnesia, anxiety, and neuropathic pain<sup>24</sup>. Anxiety is a common problem of aging and is inextricably linked with cognitive disorders<sup>25</sup>. The OFT and EPMT were used to assess the anxiety state of mice under different environmental stressors. In this study, under litter environmental pressure, there were no differences in the duration in the center during the OFT between the groups (Fig. 2), suggesting that the anxiety states of mice in the different groups were similar under mild environmental stress. Under greater environmental pressure, subacute aging mice exhibited anxiety-like behaviors (manifested as less access to the open arms), whereas dietary Met supplementation ameliorated the anxiety in these mice (Fig. 2). The different properties of the OFT and EPMT have been elucidated, and the test results may vary<sup>26</sup>. Furthermore, the NORT, YMT, and MWMT were performed to evaluate declarative memory, working memory, and spatial memory in mice, respectively. These tests necessitate interactions among various brain regions, especially between the hippocampi and prefrontal

cortex. By analyzing the behavior of mice in the NORT and YMT, subacute aging mice exhibited impaired non-spatial declarative memory (manifested as significantly decreased DI values) and working memory (manifested as a significant decrease in spontaneous alternation rate), whereas dietary Met supplementation effectively improved these cognitive impairments (Fig. 3). Moreover, cognitive dysfunction manifested as longer latency period to find the hidden platform and decreased duration and distance spent moving around the platform after its removal, was detected during the MWMT in subacute aging mice. Conversely, dietary Met supplementation effectively improved spatial memory (Fig. 3). Dietary Met supplementation also increased the levels of a variety of cognitive-function-related metabolites in the plasma, including betaine, creatine, myo-inositol, choline, glutamine, acetate, propionate, and isobutyrate, all of which showed decreased levels in subacute aging mice. These metabolites play critical roles in maintaining intracellular osmotic pressure (betaine)<sup>27</sup>, energy metabolism (glutamine, creatine, acetate, propionate, and isobutyrate)<sup>28–30</sup>, and signal transduction between neurons (myo-inositol and choline) in the brain<sup>31,32</sup>. Additional supplementation with these metabolites has shown beneficial effects on learning and memory function<sup>27–32</sup>. Collectively, these findings demonstrate that dietary Met supplementation improved cognitive dysfunction in subacute aging mice.

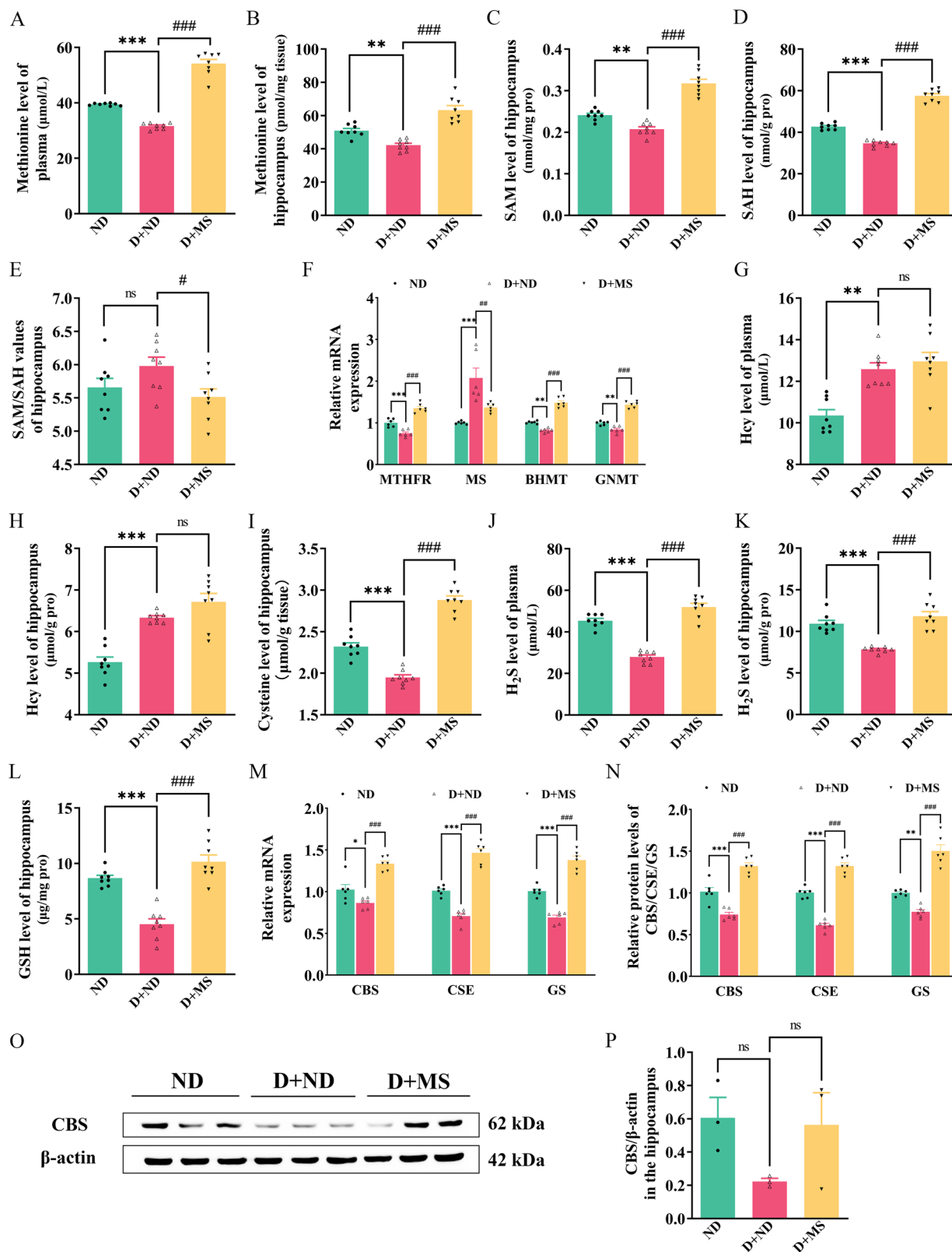
Dietary Met supplementation, which reduces oxidative stress and inflammation, contributes to improvements in cognitive function. Oxidative stress and neuroinflammation are critical factors for the occurrence and development of aging and many types of neurological dysfunction<sup>2,3,5</sup>. Excess ROS production leads to the occurrence of oxidative stress-related stimuli, additionally causing an inflammatory response, mitochondrial damage, and apoptosis in the CNS, which are hallmarks of aging<sup>3,4</sup>. In this study, dietary Met supplementation was found to significantly reduce ROS levels and enhance the resistance to oxidative stress (T-SOD and T-AOC) in the plasma and hippocampi (Fig. 6). Excess ROS or weakened resistance to oxidative stress leads to cellular redox disequilibrium, eventually promoting the production of harmful products, such as MDA and AOPPs in the hippocampi of subacute aging mice, whereas dietary Met supplementation effectively improved the redox disequilibrium in the hippocampi (Fig. 6).



**Fig. 8 | Effects of Met supplementation on the accumulation of cognitive toxins and cognition-related pathways in the hippocampi in subacute aging mice.**

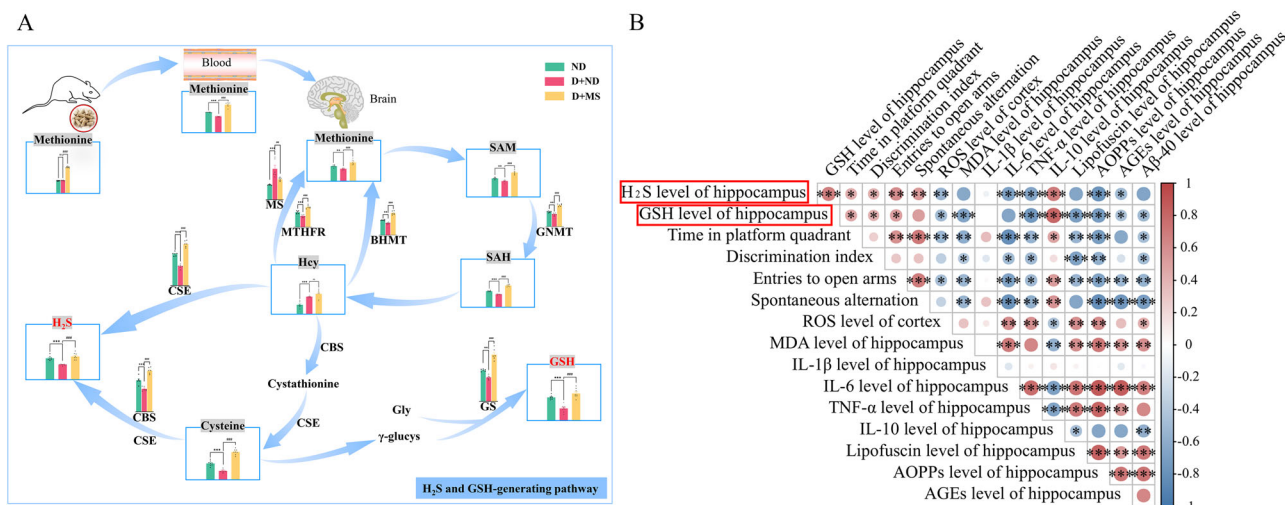
**A** AGEs, **B** lipofuscin, and **C** Aβ-40 levels in the hippocampi, **D** the relative mRNA expression levels of NF-κB, Nr2, HO-1, NQO-1, Bcl-2, caspase3, and Bax in the hippocampi, **E** the relative mRNA expression levels of BDNF, TrkB, RC3, Gap-43, PSD-95, and SYNAPO in the hippocampi, **F** the relative mRNA expression levels of

Nr1, Nr2a, Nr2b, CAMK2A, CAMK2B, and CAMK2D in the hippocampi, **G**, **H** the protein expression levels of P-NF-κB p65, NF-κB p65, BDNF, Nr2b, P-CAMK2, and CAMK2 in the hippocampi. ND: saline + normal diet; D + ND: D-Gal + normal diet; D + MS: D-Gal + Met supplementation. Data are represented as mean ± SEM ( $n = 8$  or  $6$  or  $3$ ). “ns” means not significantly different; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , versus ND; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ , versus D + ND.



**Fig. 9 | Effects of Met supplementation on the TSP in the hippocampi in subacute aging mice.** **A** Met level in the plasma, **B** Met, **C** SAM, **D** SAH, and **E** SAM/SAH levels in the hippocampi, **F** the relative mRNA expression levels of MTHFR, MS, BHMT, and GNMT level in the hippocampi, **G** Hcy level in the plasma, **H** Hcy level in the hippocampi, **I** cysteine level in the hippocampi, **J** H<sub>2</sub>S level in the plasma, **K** H<sub>2</sub>S level in the hippocampi, **L** GSH level in the hippocampi, **M** the relative mRNA expression levels of CBS, CSE, and GS in the hippocampi, **N** the relative protein

expression levels of CBS, CSE, and GS in the hippocampi, **O**, **P** the protein expression levels of CBS in the hippocampi. ND: saline + normal diet; D + ND: D-Gal + normal diet; D + MS: D-Gal + Met supplementation. Data are represented as mean ± SEM ( $n = 8$  or  $6$ ). “ns” means not significantly different; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , versus ND; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ , versus D + ND.



**Fig. 10 | The path analysis diagram of Met metabolism and Pearson's correlation analysis. A** The path analysis diagram of Met metabolism, **B** the correlation between the levels of H<sub>2</sub>S and GSH in the hippocampus with behavioral indices, and other

important biochemical indicators. (“\*”, “\*\*”, and “\*\*\*” indicate significance level of the correlation is less than 0.05, 0.01, and 0.001). ND: saline + normal diet; D + ND: D-Gal + normal diet; D + MS: D-Gal + Met supplementation.

There is a mutually reinforcing relationship between the redox disequilibrium and inflammatory response. Redox disequilibrium activates inflammatory responses, which in turn increase the degree and duration of redox disequilibrium, forming a vicious circle<sup>33</sup>. Nrf2 and NF-κB are referred to as the “master regulators” of antioxidant and inflammatory responses, and they can also modulate cognitive impairment and addiction<sup>34,35</sup>. We found that dietary Met supplementation significantly decreased the relative mRNA expression levels of Nrf2 and NF-κB as well as the phosphorylation level of NF-κB, increased the levels of downstream antioxidant enzymes HO-1 and NQO-1 in the hippocampi, normalized the levels of inflammatory cytokines in the plasma and hippocampi (IL-1β, IL-6, TNF-α, and IL-10), and further decreased the expression levels of apoptosis-related genes, including Caspase 3 and Bax (Figs. 7 and 8). Further insights into the effects of dietary Met supplementation on systemic oxidative stress and inflammatory responses can be obtained by analyzing blood metabolites. Allantoin, which is formed during the non-enzymatic oxidation of uric acid by ROS and nitrogen species<sup>36</sup>, is a promising biomarker of systemic oxidative stress. Taurine is a product of Met metabolism that has several roles in protecting against oxidative stress as a fundamental regulator of homeostasis<sup>37</sup>. N-Acetyl glycoprotein and O-Acetyl glycoprotein are typical inflammatory markers<sup>38</sup>. In this study, dietary Met supplementation regulated plasma allantoin, taurine, N-Acetyl glycoprotein, and O-Acetyl glycoprotein levels, thus further supporting the notion that Met supplementation ameliorated oxidative stress and inflammation. Oxidative stress is usually accompanied by disturbances in energy metabolism, and normal energy metabolism is essential for energy-intensive brain functions including cognitive function<sup>39,40</sup>. We found that dietary Met supplementation upregulated the levels of citrate and fumarate, and downregulated the levels of 3-hydroxybutyrate and pyruvate. These results indirectly support the idea that dietary Met supplementation ameliorates oxidative stress and inflammation. Taken together, these findings demonstrate that dietary Met supplementation abolished oxidative stress and inflammatory responses in subacute aging mice.

Reducing oxidative stress and inflammation by dietary Met supplementation alleviated cognitive decline by regulating the BDNF-TrkB pathway, improving neuronal signal transmission by regulating the NMDAR and NMDA-transmitted Ca<sup>2+</sup> influx, improving synaptic plasticity and synaptic structure, and reducing the accumulation of toxins such as Aβ (a characteristic feature of patients with AD). First, dietary Met supplementation significantly increased the relative mRNA expression levels of BDNF and its receptor TrkB as well as the protein expression level of BDNF in the hippocampi (Fig. 8). BDNF is a widely characterized

neurotrophin in mammals, mainly signaling through the TrkB receptor. It plays a critical role in the growth, development, differentiation, synaptic connectivity, and post-injury regenerative repair and protection (especially in the prefrontal and hippocampal areas, which are related to cognitive function) of neurons<sup>41</sup>. BDNF levels and BDNF-mediated signaling pathways are particularly adversely affected by oxidative stress and inflammation<sup>42</sup>. Second, NMDAR is one of the important targets for BDNF modulation<sup>43</sup>, and hippocampal long-term potentiation (LTP) requires the activation of postsynaptic Ca<sup>2+</sup> channels and NMDAR<sup>44</sup>, constituting cellular substrates of learning and memory<sup>45</sup>. A previous study demonstrated that a redox imbalance influences NMDAR activity in a way corresponding to age-related cognitive impairment<sup>46</sup>. Aging changes the intracellular redox state into a state of oxidative stress, leading to a reduction in NMDAR activity via the redox regulation of CaMK2, and GSH can rescue this effect<sup>46</sup>. We found that dietary Met supplementation increased the relative mRNA and protein expression levels of Nr2b as well as the phosphorylation level of CaMK2 (Fig. 8). Meanwhile, dietary Met supplementation significantly upregulated RC3, Gap-43, PSD-95, and SYNAPO in the hippocampi (Fig. 8). These genes encode synaptic-plasticity-related proteins (neurogranin and neuromodulin) and neurotransmission-regulating synaptic proteins (postsynaptic density protein 95 and synaptopodin), which are involved in learning and memory functions and are downregulated in response to oxidative stress and inflammation<sup>47,48</sup>. Finally, dietary Met supplementation reduced the levels of aging-related toxins, including AGEs, lipofuscin, and Aβ-40, in the hippocampi of subacute aging mice (Fig. 8). Excess AGEs and lipofuscin are produced in the aging brain<sup>49,50</sup>, causing the aggregation of proteins, the formation of Aβ, and subsequent cognitive impairment<sup>51–53</sup>. Thus, dietary Met supplementation alleviated aging-related cognitive decline, which was attributed to enhanced BDNF-TrkB signaling and neuronal signal transmission mediated by the NMDAR pathway, as well as the reduced accumulation of toxins.

Dietary Met supplementation potentially induces the abovementioned beneficial effects by increasing flux via the TSP. Met primarily participates in protein synthesis and a variety of metabolic pathways such as the TSP, Met cycle, and salvage cycle. The TSP is increasingly recognized as a “master regulator” of redox balance and integrates stress responses via the generation of several sulfur-containing metabolites, such as H<sub>2</sub>S and GSH<sup>9</sup>. H<sub>2</sub>S is capable of modulating multiple physiological processes, including responses to inflammatory stimuli, oxidative stress, and neuronal signaling pathways<sup>9</sup>. In the hippocampi, H<sub>2</sub>S selectively enhances NMDAR-mediated signaling, facilitates the induction of LTP<sup>54</sup>, and regulates Ca<sup>2+</sup> in all important brain

cell types including neurons. Thus, H<sub>2</sub>S plays important roles in the communication between neurons and glial cells and in regulating synaptic plasticity<sup>55</sup>. Furthermore, H<sub>2</sub>S supplementation has been confirmed to effectively prevent cognitive dysfunction in several models of cognitive deficiency<sup>56–59</sup>. In this study, dietary Met supplementation significantly increased H<sub>2</sub>S levels in the hippocampi and plasma (Figs. 9 and 10). Additionally, CBS and CSE are key enzymes regulating H<sub>2</sub>S production in the brain through the TSP, and they have been used as important targets to stimulate the TSP, thus reducing oxidative stress and inflammation, and improving brain function<sup>9,60</sup>. The absence of CBS and CSE can result in oxidative stress, aberrant stress responses, and cognitive impairment<sup>61,62</sup>. In this study, dietary Met supplementation increased the relative mRNA expression levels of CBS and CSE as well as the protein expression level of CBS (Figs. 9 and 10), suggesting that the increase in H<sub>2</sub>S levels was attributable to the increased levels of key H<sub>2</sub>S-producing enzymes. Just as striking as the increase in H<sub>2</sub>S levels is the increased levels of GSH as well as GS in the hippocampi after dietary Met supplementation. GSH is one of the most important antioxidants in the brain<sup>63</sup>. The redox state of the brain, largely controlled by GSH, regulates NMDAR activity, and GSH is highly relevant to aging and aging-induced learning and memory dysfunction<sup>46</sup>. A deficiency in GSH during brain development can lead to cognitive deficiencies and schizophrenia-like behavior<sup>64,65</sup>. In addition, dietary Met supplementation led to a decreased SAM/SAH ratio in the TSP (Figs. 9 and 10), which is otherwise increased in AD mice<sup>66</sup>. Moreover, MTHFR, MS, and BHMT are rate-limiting enzymes for the remethylation of Hcy to synthesize Met. In this study, dietary Met supplementation significantly increased the expression levels of MTHFR and BHMT and decreased the expression level of MS in the hippocampi (Figs. 9 and 10), implying that dietary Met supplementation activates the Met cycle, thus reducing excessive Hcy levels. Although Hcy levels in the plasma and hippocampi were higher in the D + MS group than in the ND group, increased flux via the TSP may balance the adverse effects of Hcy. Of note, a previous study showed that H<sub>2</sub>S supplementation inhibits glial activation and inflammatory responses induced by Hcy<sup>67</sup>. Amino acids are substrates for the synthesis of proteins and are essential for the renewal and repair of brain tissue<sup>68</sup>. Dietary Met supplementation increased the levels of multiple amino acids, including Met, tyrosine, isoleucine, glycine, threonine, and phenylalanine, and decreased the level of urea, the end-product of amino acid metabolism (urea) (Fig. 4). Increased utilization of Met by the body may result in the conservation of other amino acids, which, could then facilitate the brain damage repair and improve cognitive function. For example, glycine is a substrate for GSH synthesis. Taking these findings together, the dietary Met supplementation-mediated prevention of cognitive dysfunction potentially occurs by increasing flux via the TSP.

Interestingly, Met is a double-edged sword in health and disease. Both Met restriction (MR) and supplementation in moderation are beneficial, but both are harmful after excess (e.g., the dosage is not appropriate), whether restricted or supplemented<sup>14</sup>. On the one hand, Met plays an important role in initiating protein synthesis, and plays a vital role in the production of important molecules such as GSH, SAM, and H<sub>2</sub>S. Appropriate dietary supplementation of Met improves hepatic steatosis, insulin resistance, inflammation, fibrosis, bone health, and brain health<sup>13,14,19</sup>. In particular, in this study, methionine supplementation was found to improve cognitive function associated with upregulation of the TSP. On the contrary, excessive dietary Met intake is a potential risk factor for nonalcoholic fatty liver disease and a trigger factor for Alzheimer's-like neurodegeneration in normal mice<sup>69,70</sup>. Most of these studies show that Met serves major roles through its metabolism. Differences in Met dose, duration of intervention, or objects for intervention may be important factors to determine the effects. Thus, precise control of the metabolic pathway of Met is critical for maintenance of optimal cellular function, which is well worth a deeper look.

In conclusion, this study revealed that dietary Met supplementation improved cognitive dysfunction in subacute aging mice, ameliorated

oxidative stress and inflammation, selectively modulated the BDNF-TrkB pathway and neuronal signal transmission mediated by the NMDAR pathway in the hippocampi, and increased flux via the TSP. These results suggest that dietary Met supplementation prevents cognitive dysfunction associated with the upregulation of the TSP in subacute aging mice. Some animal foods (e.g., meat, eggs, and milk) as well as plant foods high in protein are rich in Met. Met supplementation in the elderly can be achieved by adjusting the daily diet or special dietary supplements. Our study provides theoretical support for the important roles of the Met metabolic pathway and suggests that appropriate dietary Met supplementation is a promising therapy to prevent cognitive dysfunction in older adults.

## Methods and materials

### Materials and animals

D-gal was obtained from Sigma Aldrich (St. Louis, MO, USA). The specific components of the diets are presented in Supplementary Table S1. S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Huabang Bioengineering Institute (Shanghai, China). Homocysteine (Hcy), cystathionine- $\beta$ -synthase (CBS), and cystathionine- $\gamma$ -lyase (CSE) ELISA kits were purchased from Huijia Biotechnology (Xiamen, China). Glutathione synthetase (GS) ELISA kit was purchased from Shengyan Biology (Beijing, China). TRIzol reagent and SYBR Green Master Mix were purchased from Vazyme (Nanjing, China). Reverse transcription kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primers were purchased from Sangon (Shanghai, China). RIPA buffer (BN25011), protease inhibitors (BN25002), and protein phosphatase inhibitors (BN25015) were purchased from Biorigin (Beijing, China). Phosphorylated nuclear factor kappa B (NF- $\kappa$ B) (P-NF- $\kappa$ B p65, Ser536, product code 310013), NF- $\kappa$ B p65 (product code 380172), brain-derived neurotrophic factor (BDNF, product code 381133), phosphorylated Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (P-CAMK2, Thr305, product code 310264), and CAMK2 (product code 380440) were purchased from Zhengneng Biotechnology (Chengdu, China). N-methyl-D-aspartate receptor (NMDAR) 2b (Nr2b, product code GB115472) and CBS (product code GB111794) were purchased from Servicebio (Wuhan, China). Malondialdehyde (MDA), total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC), and reduced glutathione/oxidized glutathione (GSH/GSSG) levels were measured using kits from Jiancheng Bioengineering Institute (Nanjing, China). Lipofuscin, advanced oxidation protein products (AOPPs), advanced glycation end products (AGEs), amyloid beta peptide (A $\beta$ -40), interleukin 1 beta (IL-1 $\beta$ ), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- $\alpha$ ), and interleukin 10 (IL-10) levels were determined using ELISA kits from Huijia Biotechnology (Xiamen, China). All chemicals and reagents used in this study were of analytical grade. Male 8 weeks old ICR mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China).

### Animal experiment design

The animal studies followed the ethical standards outlined in the Laboratory Animals Care guidelines of Jiangsu Province (China) and the 1964 Declaration of Helsinki and its subsequent amendments and were approved by the Animal Care and Use Committee of the Animal Nutrition Institute of Jiangnan University. Mice were maintained in a standard specific-pathogen-free laboratory. After acclimatization, the mice were randomly divided into various treatment groups. The mice in the control (ND group,  $n = 10$ , 0.86% Met) and model (D + ND group,  $n = 10$ , 0.86% Met) groups were fed a normal diet, whereas the mice in the intervention group (D + MS group,  $n = 10$ , 1.72% Met) were fed a Met-supplemented diet. The Met dosage was determined based on the data from several previous studies<sup>12,13,71</sup>. The ND group was administered saline subcutaneously for 11 weeks, and the D + ND and D + MS groups were administered 0.15 g/kg/day of D-Gal subcutaneously. The D-Gal dosage was decided based on the dosage used in a previous study<sup>72</sup>.

## Behavioral experiments

After 60 days of treatment, behavioral experiments were conducted in sequence (Fig. 1A). Behavioral experiments were performed as described previously with slight modifications<sup>73–77</sup>.

**Open-field test (OFT):** A square box was used as the experimental device. The mice were placed centrally and were allowed 10 min of free exploration. Mouse behavior was monitored and recorded using ANY-maze software (Stoelting, Wood Dale, IL, USA). Mice that were anxious moved less into the central area and more along the walls.

**Novel object recognition test (NORT):** On the first day, the mice were successively placed in the OFT box with no objects for 10 min. On the second day, the mice were placed in the same box containing two identical objects and allowed 10 min of free exploration. On the third day, one of the objects was replaced with a new one. The discrimination index (DI) was used to measure the ability of the mice to discriminate the new object. Mice with better non-spatial declarative memory explored new objects for longer, and had greater DI values.

**Y-maze test (YMT):** The mice were placed in a Y-maze for 8 min for free shuttling. Entering all three arms of the maze consecutively was regarded as a true alternation. The spontaneous alternation rate, calculated as (the number of true alternations)/(the total number of entries into different arms - 2) was higher for mice with better working memory.

**Elevated plus maze test (EPMT):** The experimental device contained two open and two closed arms. Mice were placed in the center for 5 min of free exploration. Mice with greater levels of anxiety entered the open arms less frequently.

**Morris water maze test (MWM):** The experimental device was a round pool with a platform. On the first day, the platform was exposed to 1 cm of liquid. On the second day, the platform was positioned 1 cm below the liquid level. For 5 consecutive days, latency was recorded during daily training trials. On the final day, the platform was removed, and the mice were allowed to swim freely for 1 min. Mice with better spatial memory showed less latency and greater motility in the target quadrant.

## Sample collection

All mice were intraperitoneally anesthetized with 1.25% avertin at the dose of 15  $\mu$ L/g body weight before being sacrificed. Blood was collected from the heart of anesthetized mice. A small amount of blood was taken to measure ROS levels according to a previous protocol<sup>78</sup>. After centrifugation, plasma was collected from the remaining blood samples for the determination of other indicators. The hippocampi and cortex were collected on ice, and stored at  $-80^{\circ}\text{C}$ .

## Plasma metabolome analysis

Plasma samples were prepared for nuclear magnetic resonance (NMR) spectroscopy using a previously described method<sup>79</sup>. Previously reported NMR detection parameters were used<sup>80</sup>. Spectra were analyzed using Mestrenova software (version 6.1.1, Mestrelab Research S.L., Spain). Initially, phase correction and baseline correction were performed for the spectra with reference to lactate resonance ( $\delta$  1.33 ppm). Subsequently, based on their chemical shifts, peak multiplicities, and relative intensities, the spectral signals were assigned to specific metabolites, as described in previous studies and the internal database<sup>69,81,82</sup>. The spectral range ( $\delta$  0.70–8.50 ppm) was divided into bins of 0.01 ppm, and the range ( $\delta$  4.68–5.00) was excluded. The resulting integral data were further processed using SIMCA-P+ (version 14.1, Umetrics, Umea, Sweden) and IBM SPSS software (version 26.0, SPSS Inc., IL, USA) based on methods used in previous studies<sup>81,83</sup>.

## Assays for TSP-related parameters

Met and cysteine were detected using high-performance liquid chromatography (HPLC, Agilent 1200SL, CA, USA) based on a previously reported method with minor modifications<sup>84</sup>. The chromatographic column for HPLC was Eclipse Plus C18 (2.1  $\times$  50 mm, 1.8  $\mu$ m, Agilent), and the flow rate was 0.42 mL/min. Mobile phase A was 10 mM  $\text{Na}_2\text{HPO}_4$  : 10 mM  $\text{Na}_2\text{B}_4\text{O}_7$ , pH 8.2 : 0.5 mM  $\text{NaN}_3$  (5.6 g  $\text{Na}_2\text{HPO}_4$  + 15.2 g  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$

were dissolve in 4 L water + 32 mg  $\text{NaN}_3$ ). Mobile phase B was acetonitrile: methanol: water (45:45:10, vol/vol). SAM, SAH, Hcy, CBS, CSE, and GS levels were measured using ELISA kits. For the determination of GS levels, the mouse GS standard and samples (10  $\mu$ L) were added to the plate pre-coated with anti-mouse GS antibody, and then another strain of horseradish peroxidase (HRP)-labeled anti-mouse GS antibody (100  $\mu$ L) was added. After incubation for 60 min at  $37^{\circ}\text{C}$  and washing, remove the uncombined components. Substrate A and B were added (50  $\mu$ L + 50  $\mu$ L), and the substrate catalyzed by HRP produced a blue product after incubation for 15 min at  $37^{\circ}\text{C}$ , which was finally converted to yellow under the action of the stop solution (2 M sulfuric acid, 50  $\mu$ L). The absorbance was measured at 450 nm.  $\text{H}_2\text{S}$  levels were determined as described by Qu et al.<sup>85</sup>. GSH levels were determined using an assay kit from Jiancheng Bioengineering Institute (Nanjing, China).

## Reverse transcription-polymerase chain reaction (RT-PCR)

TRIzol reagent was used to extract total RNA. The A260/A280 ratio was measured to determine RNA purity. Reverse transcription was performed using a commercial reverse transcription kit. A reaction mixture comprising cDNA, primers (Table S2), and SYBR Green Master Mix was prepared according to the manufacturer's instructions. RT-PCR was performed as previously described<sup>86</sup>.

## Western blotting analysis

Tissues were homogenized in RIPA buffer containing protease inhibitors and protein phosphatase inhibitors. After incubation, centrifugation, protein quantitation, adding loading buffer, and boil, western blotting was performed. Briefly, 10% SDS-PAGE was used. After electrophoresis, the protein was transferred to NC membrane. After blocking, washing, incubation with primary antibody, and washing, the membranes were incubated with a secondary antibody. Then, the membranes were washed before detection.

## Statistical analysis

Statistical analysis was performed using Student's *t*-test, one-way analysis of variance, followed by Tukey's multiple comparison test, or Tamhane's post hoc test using IBM SPSS 26.0. The results are presented as the mean  $\pm$  standard error of the mean (SEM). Statistical significance was set at  $p < 0.05$ .

## Data availability

The authors declare that the data supporting the findings of this study are available within the article.

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## Author contributions

The study was designed by Yuhui Yang, Guowei Le, and Yonghui Shi. The experiments and statistical analysis were conducted by Yuncong Xu, Yuhui Yang, and Bowen Li, and supervised by Yuhui Yang and Yanli Xie. The article was written by Yuncong Xu, and modified by Yuhui Yang.

### Competing interests

The authors declare no competing interests.

### Additional information

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