Supplementary Material

Pterostilbene Improves Cognitive Performance in Aged Rats: An *in Vivo* Study

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Materials and Methods

In vitro **treatments:** Pt was purchased from Waseta Int. Trading Co. (Shangai, P.R.China). The compound was dissolved in sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich). Stock solutions were prepared at 1000× the desired working concentration (25μM) in order to fix the final DMSO percentage at 0.1% in all cases (including controls without drugs). Experiments were performed in HEK293 cells. They were maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich), supplemented with 10% Fetal Bovine Serum (FBS, Euroclone) and 1% Penicillin/Streptomycin, and grown at 37°C, 5% CO_2.
HEK293 cells were seeded in standard 24-well plates and allowed to attach overnight in complete DMEM medium. The following day cells were serum-starved for approximately 16 hours before treatment with pterostilbene. At the end of the treatments, the medium was removed and 150 μl di Lysis Buffer (25 mM TRIS pH 7.8 + 2.5 mM EDTA + 10% glycerol + 1% NP40 + 2 mM DTT + 1% phosphatase inhibitors cocktail 2 and 3 (Sigma-Aldrich) + 1% protease inhibitors cocktail (Sigma-Aldrich)) were added to each well. The plates were then frozen at -80°C. Total extracts were collected from each well after scraping, vortexed for 10 s and then centrifuged at 20000 *g* for 10 minutes at 4°C. To enhance protein separation, samples were solubilized for 30 min at RT in Sample Buffer (10% glycerol + 42 mM Tris/HCl pH 6.8 + 3% SDS + 33 mM DTT + Bromophenol blue) and then heated for 5 minutes at 70°C.

Animal handling and Pt administration: all procedures met the requirements of Italian law (Laws 116/92 and 26/14) and of the European Community Council (Directives 86/609/EEC and 210/63/UE) and were approved by the Italian Ministry of Health (DM n. 282/2013-B). 9 male and 6 female 18-month-old Sprague Dawley rats were housed in the facility of the Institute of Neuroscience (Pisa) under a 12-hour light regimen. Food and water were provided *ad libitum*.

Rats were subdivided into Pt- or vehicle-treated groups; females and males were equally distributed. Animals belonging to the treated group received for 20 consecutive days a 22.5 mg/kg dose of Pt in a palatable jelly twice a day at 12-hour intervals. Instead, animals of the control group received "unloaded" jelly.
Drug incorporation in jelly was preferred over addition in food or water to provide Pt as it ensures a controlled administration and does not cause stress to animals [1].
To manufacture the jelly, two different solutions were prepared in parallel:

- Grated Parmigiano cheese was blended with tap water (ratio 1:2) under stirring for 30 minutes; then the mix was filtered in order to obtain a savory liquid
- Gelatine (Paneangeli) was re-hydrated with water at room temperature for 10 minutes; following re-hydration, it was liquefied by heating at 55-60°C under stirring for 10 minutes

Finally, the two liquids were mixed to obtain a homogenous solution. Pt was added to the mixture for the treated group. The difference in the average weight of male and female animals was taken into account in calculating the concentration of Pt to provide. The preparation was further stirred and aliquoted into a 48

well-plate and stored at 4°C. Before the beginning of the Pt treatment, animals were handled daily to avoid neophobia. Rats promptly learned to voluntarily eat jellies.

Behavioral tests: The cognitive performance of the animals was evaluated both prior and after 20 days of jelly administration. We used a working memory test (T-maze) and two declarative memory tests (visual object recognition (vORT), and object-in-context (OCT)), as described below. As their execution requires several days, vehicle jellies were provided to all animals for the entire duration of baseline assessment to avoid loss of interest in the flavor. Similarly, we prolonged the administration of "unloaded" and "loaded" jellies to untreated and treated animals respectively, during the period of final evaluation (about two weeks) to sustain the effect of Pt, if any.

- *T-maze test:* the T-maze test was carried out adapting the protocol reported by Deacon and Rawlins [2]. Animals were placed at the bottom of a T-shape box for ten successive trials at 90 second intervals. Each time the animal chose and entered the arm opposite to the one examined during the previous trial, this was classified as an alternation response. The alternation rate was calculated by considering the sequence of nine successive trials and expressed as the mean of the arm alternation.
- *Visual Object recognition test (vORT):* this test was carried out using a polyvinyl chloride (PVC) square arena, with black walls and white floor, and cubical plastic objects that differed for the visual patterns lining the walls of the cube. The experimental protocol was set up according to the guidelines described by De Rosa et al. [3]. The entire assessment consisted of three consecutive sections: 1) habituation phase; 2) sample phase; 3) test phase. In the first phase, rats were placed in the empty arena for 5 minutes in order to familiarize with the apparatus and test room; in the second phase, after about 24 hours, each animal was placed in the arena again, exposed to two identical objects for 5 minutes and then returned to its own cage; the third phase was performed either 1 or 24 hours after the sample phase. The rats were placed back in the arena for a third time and exposed to one familiar object (an object identical to those seen in the sample phase) and a novel object, located in the same positions as in the sample phase, for 5 minutes. To avoid possible preferences for one of the two objects, the choice of the new and old object and the position of the new one was randomized among animals. The time spent by each animal exploring the objects was recorded and a discrimination index was calculated as described below (see measurements and statistics section).
- *Object in context test (OCT):* the protocol used was similar to that previously described by De Rosa et al. in 2005 [3]. Two different PVC arenas were used. The first one presented horizontal white stripes on the black walls and a floor covered by rough Plexiglas. The second one had gray walls and the floor was made by smooth Plexiglas. The first and second arena represented experimental conditions A and B, respectively. The assessment consisted in determining the animals' sensitivity to a change in context for a given object. In this case, two 5 minute-long habituation phases were carried out during which rats were exposed to both conditions A and B. The two habituation phases were followed by four sample phases and one test phase, each lasting 3 minutes. The recovery period within the sample phases was 3 minutes long while there were five minutes of interval between the

last sample phase and the test phase (the habituation phase was started two days before the test phase). During the four sample phases, two objects were positioned in adjacent corners of the arena. Specifically, objects A1 and A2 were placed in environment A during phases 1 and 4 while objects B1 and B2 were placed in environment B during phases 2 and 3. Finally, the test phase took place in the same environment of sample phase 4, but object A2 was replaced by object B2 so that only one object was in the same environment as in the sample phase. To avoid any confounding preference for context A or context B, half of the animals started the sample phase in environment A with object A1 and A2 and finished the assessment in the same environment with objects A1 and B2 and *vice versa*. Habituation and samples phases will be referred to as a single training period in the results section.

Boxes and objects were cleaned up between each trial to avoid interference by olfactory suggestions.

After the final round of behavioral tests, the animals were sacrificed under anesthesia (Isoflurane). The brains were quickly removed and DG, hippocampus and prefrontal and perirhinal cortices were dissected. Each specimen was rapidly frozen by immersion in a bath of isopentane and dry ice and stored at -80°C.

Measurements and Statistics: The mean of the arm alternations was calculated as evaluation criterion for the T-maze experiments. The preference to enter one arm rather than the other one was compared across groups (treated and untreated) with the Two-way repeated measures (RM) ANOVA and the Holm-Sidak method for post hoc comparison.

For the statistical analysis of the results of vORT and OCT, the parameter taken into account was the time spent exploring the objects. The exploration of an object was defined as directing the nose to the object at a distance of < 2 cm and touching it with the nose. Turning around, climbing over, or sitting on the object were not considered. Animals were excluded if their exploration time was < 3 seconds in the sample phase. Similarly, they were discarded if the time exploring familiar and new objects was < 1 second in the test phase. A discrimination index (DI) was calculated, both for vORT and OCT, as the difference between the time spent exploring new and old objects divided by the total time spent exploring the objects [(n - f)/(f + n), where n represents new and f represents familiar]. The indexes calculated were used for comparison across different groups (treated and untreated) with the Two-way repeated measures ANOVA and the Holm-Sidak method for post hoc comparison.

RT-qPCR and Western Blotting: total RNA and proteins extraction was obtained from samples of the cerebral areas selected following TRIzol (Invitrogen) extraction protocols, according to the manufacturer's instructions. Reverse transcription was performed using SuperScript VILO cDNA Synthesis kit, starting from 400 ng of RNA. Reaction tubes were shaken and incubated first at 25°C for 10 minutes. Then, they were warmed up at 42°C for 60 minutes. Finally, the reaction was terminated at 85°C for 5 minutes. To measure the expression levels of genes of interest, specific primers were designed (see Supplementary **Table S1**). IQ Syber Green Supermix (Biorad) was used as detection system. Relative quantification of target genes was performed using the mathematical model described in [4]. GAPDH was used as reference gene. For

Western Blotting, proteins were separated by SDS-PAGE (Pre-cast NuPAGE Bis-tris Gels, Invitrogen). After electrophoretic separation, proteins were transferred to PVDF membranes (Immobilion-FL). The membranes were saturated with 5% BSA (Sigma-Aldrich) in TBS-T buffer (TBS supplemented with 0.1% Tween-20, Sigma-Aldrich) for 1 hour and then incubated with primary antibodies (complete list reported in Supplementary **Table S2**) at 4°C overnight. Odissey Imaging equipment (LICOR) was used as detection system.

Table S1. Detailed list of transcripts analyzed.

DG: dentate gyrus; Hipp: hippocampus; PFC: prefrontal cortex; PRC: perirhinal cortex.

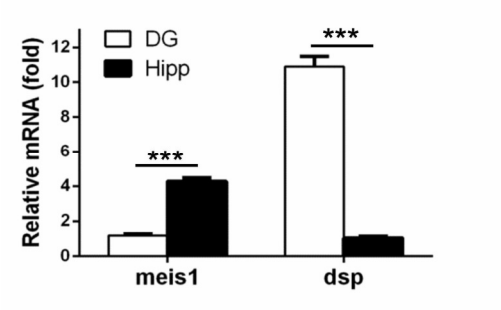
Gene	Cerebral areas analyzed				Primer sequence	
	DG	Hipp	PFC	PRC	Fw	Rv
bdnf	X	X	X	X	ATAGGAGACCCTCCGCAA	CTGCCATGCATGAAACACTT
dsp	X	X			CACTCCCAGTCTTCACAGCA	TTTCTCCAGGTCCCACAATC
dlg4	X	X	X	X	GAGTGCTTCTCAGCCATCGT	ATGTAGGGGCCTGAGAGGTC
gapdh	X	X			ATCACCATCTTCCAGGAGCG	GATGGCATGGACTGTGGTCA
meis1	X	X			GCACAGGTGACGATGATGAC	GAAGGATGGTGAGTCCCGTA
nr4a2	X	X			GCTGAAGCCATGCCTTGTGTT	TCGGAGCTGTATTCTCCCGA
rbbp4	X	X	X	X	GCTCCCTAATGACGATGCTC	ATGATGCAAGGGTTCTGAGG
rest	X	X	X	X	AACTCACACAGGAGAACGCC	TGTGAACCTGTCTTGCGTGT

Table S2. Complete list of proteins analyzed.

DG: dentate gyrus; Hipp: hippocampus; PFC: prefrontal cortex; PRC: perirhinal cortex.

Protein	Cerebral areas analyzed				Source
	DG	HIPP	PFC	PRC	
β-Actin	X	X	X	X	Cell Signaling
BDNF	X	X		X	Abcam
Drp1	X				BD Transduction Laboratories
Fis1	X	X			Adipogen
H3	X	X	X	X	Cell Signaling
H3K9	X		X		Cell Signaling
Mnf1	X		X		Santa Cruz Biotechnology
pCREB (S133)	X	X	X	X	Cell Signaling
PSD-95	X	X	X	X	Abcam
RbAp48	X	X	X		Abcam
REST	X	X	X		Millipore
VDAC1/Porin1	X	X	X		Abcam

Figure S1. **Accuracy of DG dissection.** RT-qPCR of dentate gyrus and hippocampal markers (see text). ***: $p < 0.001$; statistical analysis performed with the two-tailed unpaired t-test. N=15. Error bars reported as + SEM.



We checked the accuracy of DG isolation measuring the expression levels of the *dsp* and *meis1* genes, molecular markers of the DG and the rest of the hippocampus, respectively [5]. In the whole set of animals the mRNA levels of the *dsp* gene were in fact much higher in the DG samples than in the residual hippocampal fraction. *Vice versa*, the *meis1* transcript was more abundant in the non-DG hippocampal tissue.

References

1 Zhang L: Voluntary oral administration of drugs in mice. Protocol Exchange 2011

2 Deacon RM, Rawlins JN: T-maze alternation in the rodent. Nature protocols 2006;1:7-12.

3 De Rosa R, Garcia AA, Braschi C, Capsoni S, Maffei L, Berardi N, Cattaneo A: Intranasal administration of nerve growth factor (NGF) rescues recognition memory deficits in AD11 anti-NGF transgenic mice. Proceedings of the National Academy of Sciences of the United States of America 2005;102:3811-3816.

4 Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research 2001;29:e45.

5 Hagihara H, Toyama K, Yamasaki N, Miyakawa T: Dissection of hippocampal dentate gyrus from adult mouse. Journal of visualized experiments : JoVE 2009