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Long-term administration of resveratrol at low doses improves neurocognitive performance as well as cerebral blood flow and modulates the inflammatory pathways in the brain

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Abstract

There is an increasing prevalence of coincident cerebrovascular deficiency and cognitive dysfunction with aging. Increased oxidative stress as well as inflammation that occurs with aging are associated with the impairment of cerebral vascularization. Interestingly, Resveratrol (RSV), a natural phytoalexin, is known to be a strong antioxidant and possesses anti-inflammatory properties. Collectively, these observations strongly suggest that RSV could protect against cerebral vascularization defect and then improves the decline cognitive function associated with aging. In order to test this hypothesis, we investigated the effect of a long-term RSV treatment (1.25 mg/day for 5 months) on cognitive performances of animals that we have allowed to age normally. Then, we further analyzed the gene expression profile and the cerebral blood flow in the brain. By means of novel object recognition (NOR) test, we observed that RSV enhanced NOR performances of aged rats. In addition, RSV enhanced cerebral blood flow during NOR task in aged rats. Using microarrays experiments, we also showed that several pathways related to inflammation and oxidative stress (Eicosanoid signaling, MIF-mediated innate immunity, NF- κ B signaling, TNFR2 signaling, IL6 signaling, Production of nitric oxide and ROS) were down-regulated in the brain of RSV treatments rats compared to control rats. In conclusion, these results support that a long-term treatment with RSV improves cognitive performance in the elderly male rat model. This effect is associated with an increase in cerebral blood flow and a decrease in the expression of several pro-inflammatory pathways in the brain.

Keywords: Resveratrol; cerebral blood flow; inflammation; cognition

1. Introduction

Resveratrol (3,5,4'-trihydroxy-trans-stilbene; RSV) is a natural phytoalexin produced by various plants such as the red grapes, peanuts, berries, and *Polygonum cuspidatum*, that exerts multiple beneficial metabolic actions *in vivo* [1,2]. The beneficial properties of trans-RSV are extensively depicted in the literature and including action on the arylhydrocarbon receptor, kinases inhibition, anti-inflammatory, antioxidant, analgesic and anti-tumoral activities [3–5]. Protective effects against cardiovascular and neurodegenerative diseases as well as metabolic effects on bone and glucose metabolism homeostasis have also been described [1–4,6–10]. In the inflammation context, RSV has anti-inflammatory properties by inhibiting NFκB, COX and AP-1-dependent inflammatory processes, resulting in reduction of levels of IL-1, TNFα and other inflammatory cytokines [5,10–12]. To date, it has also been shown that RSV can act as antioxidant compound through its ability of either directly neutralizing reactive oxygen species/reactive nitrogen species (ROS/RNS) or indirectly upregulating the expression of cellular defensive genes [13]. In particular, RSV stimulated the expression of antioxidant defensive enzymes such as catalase as well as superoxide dismutase [13].

Preclinical evidence supports the notion that RSV may play a role in the treatment and prevention of neurodegenerative diseases, such as Huntington's disease, Parkinson's disease, and Alzheimer's Disease (AD) [14,15]. For instance, several studies suggest that RSV protects neurons from ROS and RNS, hydrogen peroxide free radicals, amyloid β (Aβ) and other intra- and extracellular toxins and insults associated with neurodegenerative disorders [15–17]. In this context, it has been suggested that RSV promote these protective effects through the sirtuin 1 (SIRT1) pathway. In fact, RSV intervention is associated with higher gene expression and serum concentrations of SIRT1 in rodents and humans [8,18,19].

Normal aging is associated with modifications to the biomechanical properties of blood vessels. These modifications are involved in anatomical and functional alterations in the brain

vasculatures and potentially lead to hypoperfusion and/or neurodegeneration [20,21]. Actually, the relationship between a decrease of cerebral blood flow (CBF) and a decline of neuronal functions is not clear. Nevertheless there is an increasing prevalence of coincident cerebrovascular deficiency and cognitive dysfunction with aging that is also well recognized in AD [22–25]. Interestingly, different studies have shown that increased concentrations of SIRT1 are associated with better vascular homeostasis and metabolic profile and protection against endothelial senescence [26,27]. In addition, RSV improved vascular injuries by modulating SIRT1 but also nuclear factor-erythroid 2–related factor 2 (Nrf2) [28].

Collectively, these observations strongly suggest that RSV can protect against cerebral vascularization defect and then improves the decline cognitive function associated with aging. However, this effect of RSV on cognitive impairment associated with blood brain flow remains to be clarified. And particularly, the molecular pathways associated with this potential effect of RSV on cognitive functions are not clearly identified. As mentioned before, inflammation and ROS has been shown to contribute to the endothelial dysfunction of the blood-brain barrier that could be involved in the induction of several nervous pathogenesis [29]. In this work, we explored for the first time that a low dose of RSV can impact the expression of main genes of the inflammation and ROS pathways. In addition, we investigated the long-term effect of RSV treatment on cognitive performances of aged rats associated with the blood flow in the brain.

2. Methods

2.1. Animal experiments and treatment

All procedures using animals were conducted according to European Union Directive 2010/63/EU and were approved by the institution's Animal Care and Use Committee (project #14191, CE7114, Aix-Marseille University). Six-month-old Sprague-Dawley rats were purchased from Janvier Labs and quarantined for ten days upon delivery. With this protocol, we want to observe the effect of RSV on gene expression, brain flow and cognitive function in older animals and not in young animals. Animals were cared by qualified operators in an accredited lab, housed in adequate-sized cages, enriched with hay agglomerates and cocoons, placed in a quiet, temperature- and hygrometry-controlled room with 12-hour light and dark cycles, daily monitoring, with access to water and a standard commercial diet ad libitum. One group of rats has been treated for five months with 50 mg/L RSV diluted in autoclaved drinking water bibs (RSV, n=5). A second group of rats had access to normal, autoclaved drinking water containing the same polysorbate 20 and polyglyceryl-3-dioleate quantities without RSV (Control, n=5). The RSV low dose used in this study was 1.25 mg/rat/day for 5 months. This protocol has been already used in several studies [30,31].

The RSV formulation consisted of natural t-Res powder (50 mg) dissolved in a complex mixture notably containing polysorbate 20 and polyglyceryl-3-dioleate as described in patent WO YVERY N 2010/007252 [32]. This solution was mixed to water (1L).

2.2. Radiotracers

Hexamethylpropyleneamine oxime kit (HMPAO, Cerestab®, General Electrics) was radiolabelled with fresh [^{99m}Tc]TcO₄⁻ pertechnetate solution (500MBq/1mL, Tekcis®, Curium) by a qualified operator, following the manufacturer's instructions.

2.3. Novel object recognition test combined with cerebral blood flow microSPECT/CT

The animal's exploratory behavior and its non-spatial learning and memory capacities were assessed with novel object recognition (NOR) test [33,34]. Briefly, the rat was placed in a black, open-field arena (100*100cm). Each animal's route was video-recorded and analyzed with EthoVision XT7 software. The day before the test, animals received a habituation session of 30 minutes in the open field. On the testing day, each rat completed four sessions of 3 minutes (with a 30-minute intercourse). During the first three sessions, the rat was allowed to explore the arena, which contained two identical objects. Total exploration time and frequencies of exploration of both objects was noted. Right before the fourth NOR session, one of the original objects in the arena was replaced by a novel different-shaped object, and 20.9 ± 2.8 MBq of [^{99m}Tc]Tc-HMPAO were injected to the rat through the tail vein. Then the rat was returned to the arena and was allowed to explore both novel and familiar objects for 3 minutes. The "object exploration" was considered when the rat sniffed at the novel object within a 1 cm-distance or touched the novel object with its nose. The time spent on each object exploration and the frequencies of exploration were video-recorded and discrimination ratios were calculated (time spent to explore the novel object compared to the total exploration time, frequency of the novel object exploration compared to the frequency of exploration of both objects). The latency period to the first exploration of the novel object was also measured. For note, the cognitive test was performed by a sole, blind and experimented zootechnician who regularly visited, cared about, and habituated the rats to prehension and manipulation.

Thirty minutes after [^{99m}Tc]Tc-HMPAO injection, the rat was anesthetized with 1.5% sevoflurane and a cerebral microSPECT/CT imaging was acquired for 20 minutes on a calibrated and qualified NanoSPECT/CT+ camera (Mediso) to assess CBF. Image analysis was performed using the 3D-ROI module part of InVivoScope® software v2.0p4 (InviCRO).

A volume of interest (VOI) was drawn over the whole brain for each animal in the axial section. Radioactivity inside each VOI was quantified and corrected by the tissue volume (MBq/ μm^3). Image color scales were normalized in order to illustrate CBF.

RNA extraction, hybridization arrays and microarrays data Analysis (IPA)

Total RNA was extracted from brain using TRIzol reagent (Thermo Fischer Scientific, Les Ulis, France) as previously described [35]. 5 rats of the control group and 5 rats of the RSV group were used.

RNA quality control was performed on an Agilent 2100 Bioanalyzer (Massy, France), according to the manufacturer's instructions. RNA was hybridized to the Agilent Whole Rat Genome (Massy, France). All labeling, hybridization, washing and scanning were performed as described in the manufacturer's protocol and as previously reported [36–38]. The arrays were scanned using an Agilent Scanner (Massy, France). The data were extracted using Agilent Feature Extraction v10.5.1.1 software and analyzed with Agilent GeneSpring GX v11.0.2 software (Massy, France).

Differential gene expression (with *p*-value adjusted < 0.05) were used in the IPA software to identify the canonical pathways differentially impacted by the RSV treatment.

Statistical analysis

Values were reported as mean \pm sd unless otherwise indicated. All data were successfully tested for normality using a Shapiro-Wilk test and then analyzed with two-tailed, unpaired t-test. Correlation was tested using Pearson test. Statistical analyses were performed with Prism® v8.4.2 (GraphPad Software). A *P* value inferior to 0.05 indicated statistical significance.

Results

Resveratrol enhanced novel object recognition performances of aged rats

Chronic RSV treated-group significantly increased the time spent on exploring the novel object compared to the control group (RSV: $89.8 \pm 6.3\%$, $n=5$; Control: $0.89 \pm 0.03\%$, $n=5$; $*P=0.0442$; Figure 1A). The mean latency to first exploration of the novel object was also significantly reduced in RSV-treated rats compared to the control group (RSV: $34.2 \pm 8.4s$, $n=5$; Control: $93.8 \pm 29.1s$, $n=5$; $*P=0.0477$, Figure 1B bottom), meanwhile no significant change was found on the usual object (RSV: $51.5 \pm 21.9s$, $n=5$; Control: $57.6 \pm 38.0s$, $n=5$; $P=0.8868$, Figure 1B top).

Resveratrol enhanced cerebral blood flow during NOR task in aged rats

A significantly higher cerebral [^{99m}Tc]Tc-HMPAO μSPECT signal was found in the RSV-treated rats ($0.66 \pm 0.11\% \text{ID}/\mu\text{m}^3$, $n=55$) compared to the control rats ($0.36 \pm 0.09\% \text{ID}/\mu\text{m}^3$, $n=5$, $**P=0.0014$, Figure 2). The injected doses of [^{99m}Tc]Tc-HMPAO were not significantly different between both conditions (respectively $22.4 \pm 3.2 \text{MBq}$ and $19.5 \pm 2.5 \text{MBq}$, $P=0.1452$).

Cerebral blood flow improvement correlated with NOR performance in aged rats

Interestingly, a significant positive correlation was found between the cerebral [^{99m}Tc]Tc-HMPAO μSPECT signal quantification and the latency to first time exploring the novel object (Figure 2C, *Pearson* $R^2=0.6547$, $**P=0.0046$).

Resveratrol treatment modified brain transcriptome

To study the global effect of the RSV treatment, microarrays experiments were performed with RNA extracted from the brain of the control and treated groups. A large number of genes were differentially regulated between the 2 groups: 7756 genes differentially regulation (p -

value adjusted <0.05, without Fold change filter), including 2790 down-regulated genes and 4966 up-regulated genes (Table 1). All these data were analyzed using Ingenuity Pathway Analysis (IPA) software. This analysis revealed that several pathways related to inflammation (Eicosanoid signaling, MIF-mediated innate immunity, NF-kB signaling, Dendritic cell maturation, TNFR2 signaling, IL6 signaling, Production of nitric oxide and ROS) were overrepresented with a negative Z-score, meaning that those pathways were down-regulated in RSV treatments rats compared to control rats (Table 2). At the opposite, several pathways were overrepresented with a positive Z-score (including, antioxidant action of vitamin C, iNos signaling, OX40 signaling), meaning that those pathways were up-regulated in RSV treatments rats compared to control rats (Table 3).

In order to know the polarization states of microglia, we analyzed genes known to be involved in M1 and M2 polarization [39]. In RSV-treated rats, there was significant up-regulation of various microglial M2 polarization markers genes, for example *IL-4*, *IL-10*, *IL-13*, *IL-25*, *CD206* and *IGF-1* (Figure 3, *p*-value adjusted <0.05, without Fold change filter). Among the down-regulated genes, some are markers of M1 polarization as *IL-23*, *TNF α* and *IFN α* (Figure 3, *n* (*p*-value adjusted <0.05, without Fold change filter)).

Discussion

In our study, we observed that long-term treatment with RSV improved cognitive performance in the elderly male rat model. This effect was correlated with an increase of CBF and associated with a decrease in the expression of several pro-inflammatory pathways in the brain.

Most studies in the literature have been performed in chemically-induced cognitive disorders models (*i.e.* with streptozotocin or isoflurane) [40–42]. The originality of our model relied on exposing animals allowed to age normally, to a 5-month RSV treatment. All animal and human studies concur on the poor bioavailability of RSV through low uptake and extensive metabolization. In humans and rodents, RSV rapidly undergoes sulfatation and glucuronidation resulting in 1% of the oral dose being observed as free RSV in blood plasma [43]. The active concentration of RSV lays in the micromolar range in most experimental models [44]. Our laboratory previously resolved this poor bioavailability issue by formulating an innovative galenic system enabling the administration of a lower dose, as used in the present study (1.25 mg/day/rat) compared to doses used in the literature for preventing cognitive defects in rats (30-100 mg/kg per day) [32,45].

It has been demonstrated that SIRT1 knock-out mice has memory defect and reduction in neurite length in hippocampus suggesting an essential function for SIRT1 in cognitive function and synaptic plasticity [46]. As one of the most powerful and selective SIRT1-activating compounds, the beneficial effects of RSV on senescence do extend to anti-inflammatory, anti-apoptotic, and anti-oxidative properties, also promoting neuroprotective autophagy and synaptic plasticity [45]. Thus, RSV has been extensively studied in preclinical cognitive decline models. In our study, chronic RSV treatment in 6-month-aged rats for 5 months resulted in enhanced performances in the NOR test, translating a gain on non-spatial working memory and exploratory capacities compared to control animals. Among the few

studies led in aged rodents investigating its effects on cognition, RSV improved both long-term memory and long-term potentiation in 8-9-month-old mice and was shown to rescue age-related neurovascular coupling in 24-month-old mice via down-regulation of NADPH oxidase-derived ROS production [40,47]. RSV prevented post-operative cognitive dysfunction in 19-22-month-old rats [48]. It has also been shown that SRT1720, a SIRT1 agonist, reduced cognitive decline in a type 2 diabetes mellitus rat model [49]. This observation was associated with an increase of peroxisome proliferator-activated receptor γ expression in hippocampus. Interestingly, we also detected an up-regulation of PPAR signaling in brain of RSV-treated animals compared to control rats during bioinformatics analysis of microarrays experiments. Our results are consistent with the literature, but the majority of preclinical studies on rodents have focused on the independent assessment of cognitive functions and vascularization of the brain. For the first time, the relation between CBF and cognitive improvements has been studied and highlighted a significant correlation.

Indeed, [^{99m}Tc]Tc-HMPAO is a clinical radiotracer of brain metabolism, whose distribution in cerebral parenchyma is proportional to CBF until trapped into the intracellular compartment. [^{99m}Tc]Tc-HMPAO SPECT therefore represents a routinely used, direct method for CBF assessment in patients but also in many preclinical rodent studies [50]. In our study, [^{99m}Tc]Tc-HMPAO was originally injected right prior to the final task of NOR test, so that the distribution phase of the radiotracer occurred during the neurocognitive task completion. We observed a significantly elevated CBF in RSV-treated aged rats during NOR compared to control rats. Interestingly, it has been shown that neurovascular endothelial SIRT1 potentiation upregulated the nitric oxide system and countered cerebral hypoperfusion injury [51]. In accordance with this study, an up-regulation of iNOS expression in brain has been also observed in the microarray analysis. This was corroborated by a recent study that evaluated a daily 10 mg/kg *p.o.* RSV treatment for 4 weeks *versus* placebo in 250-350 g rats

and confirmed a significantly reduced reactivity of resistant arteries isolated from RSV-treated animals and a significant increase in brain-derived neurotrophic factor (BDNF) serum concentrations [52]. Additional work suggested that RSV effects are likely to be mediated through reduced expressions of micro-RNA (miR-134 and miR-124), which may in turn up-regulate CREB levels to subsequently promote BDNF synthesis [40]. In another study, it has been shown that RSV promoted the M2 polarization of microglia and reduced neuroinflammation by inhibiting miR-155 [53]. In accordance with these observations, we also observed that several genes, as *interleukin 4*, *10*, *13*, *25*, *IGF-1* and *CD206*, associated with M2 polarization are up-regulated in RSV-treated animals. But these data are obtained by treatment after cerebral ischemia. In our study, long-term and low dose administration of RSV improves CBF that could rely with the anti-inflammatory and antioxidant effects of RSV.

As mentioned above, RSV is known to be a strong antioxidant and possesses anti-inflammatory properties. It inhibits NF- κ B and AP-1-dependent inflammatory processes, resulting in reduction of levels of IL-1, TNF α and other inflammatory cytokines [5,10]. Interestingly, microarray analysis indicated that NF- κ B as well as IL6 signaling were down-regulated in RSV-treated animals compared to control rats. We also detected a decrease of eicosanoid signaling. Eicosanoid signaling, similar to cytokine signaling, has been viewed as primarily a pro-inflammatory component of the innate immune response [54]. This observation is in accordance with a previous study showing that RSV decreased 8-iso-PGF2 α production both basally and after hydrogen peroxide-induced oxidative stimulus in the brain of aged rats [55]. In the context of anti-oxidant effect of RSV, a down-regulation of ROS and NOS production were detected. Through SIRT1 activation, RSV may protect neurons from ROS and NOS [13]. Our results are comparable with the work of Liu *et al.* that showed an higher level brain activity of superoxide dismutase and glutathione peroxidase in a senescence-accelerated mice treated with RSV (25, 50, 100 RSV mg/kg/day for 8 weeks)

[56].

Translation to human seems promising, as far as a recent clinical study including 80 post-menopausal women aged 45–85 years notably showed that a 14-week, daily 150 mg RSV treatment significantly enhanced both cerebrovascular function and cognition compared to placebo [57]. To date, no other terminated published clinical study focused in elder patients and evaluated its effects on CBF and/or cognition [58].

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Figures and Legends

Figure 1: Resveratrol treatment enhanced performances of senescent rats with the novel object recognition test

A: the total time spent on exploring both novel and usual object was not significantly affected by RSV treatment (RSV: 2.88 ± 0.84 , $n=5$; Control: 3.53 ± 1.01 , $n=5$; $P=0.6274$, top), although the time spent on exploring the novel object reported to the total time spent on exploring both objects was significantly elevated in RSV-treated rats (RSV: 89.8 ± 6.25 , $n=5$; Control: 38.9 ± 20.1 , $n=5$; $*P=0.0416$, bottom). B: no significant change was found on the mean latency to first exploration of the usual object (RSV: 51.5 ± 21.9 s, $n=5$; Control: 57.6 ± 38.0 s, $n=5$; $P=0.8868$, top), although the mean latency to first exploration of the novel object was significantly reduced in RSV-treated rats (RSV: 34.2 ± 8.4 s, $n=5$; Control: 93.8 ± 29.1 s, $n=5$; $*P=0.0477$, bottom).

Figure 2: Resveratrol treatment elevated cerebral blood flow during the novel object recognition test

Representative μ SPECT/CT images (cerebral window in both transversal and sagittal planes) of control animals (A, top left) and RSV-treated animals (A, top right). A significantly higher cerebral [^{99m}Tc]Tc-HMPAO μ SPECT signal quantification was found in the RSV-treated rats ($0.66 \pm 0.1\%$ ID/ μm^3 , $n=5$) compared to the control rats ($0.36 \pm 0.1\%$ ID/ μm^3 , $n=5$, $**P=0.0014$, A, bottom). Although no significant correlation was found between the time spent on the novel object and the cerebral [^{99m}Tc]Tc-HMPAO μ SPECT signal quantification (B, *Pearson* $R^2=0.3360$, $P=0.0791$), a significant correlation was found between the latency to first time exploring on the novel object and the cerebral [^{99m}Tc]Tc-HMPAO μ SPECT signal quantification (C, *Pearson* $R^2=0.6547$, $P=0.0046$).

Figure 3: Transcriptional profiling revealed the up-regulation of M2 and down-regulation of M1 microglia markers.

Volcano plot displaying the log2 fold change (x axis) against the $-\log_{10}$ adjusted p-value (y axis) for all genes differentially expressed between control and RSV-treated animals. Black data points represent significantly differentially expressed genes in brain of RSV treated vs control rats (adjusted p-value < 0.05 and $\log_2\text{FC}(\text{RSV}/\text{ctl})$).

Table 1: Numbers of regulated genes

	Without FC	Abs FC between 1 and 1.5	Abs FC up to 1.5
Total	7756	4775	2981
Up-regulated	4966	3255	1711
Down-regulated	2790	1520	1270

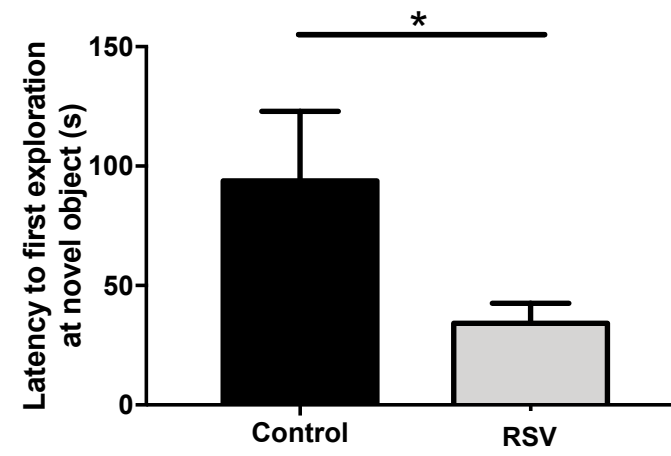
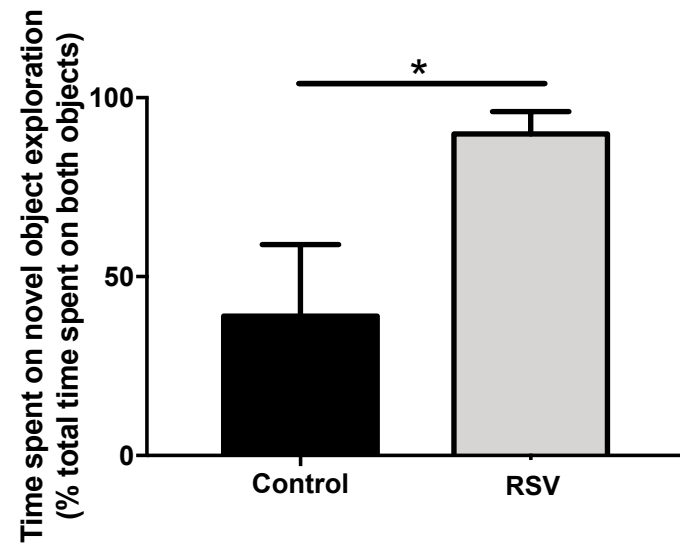
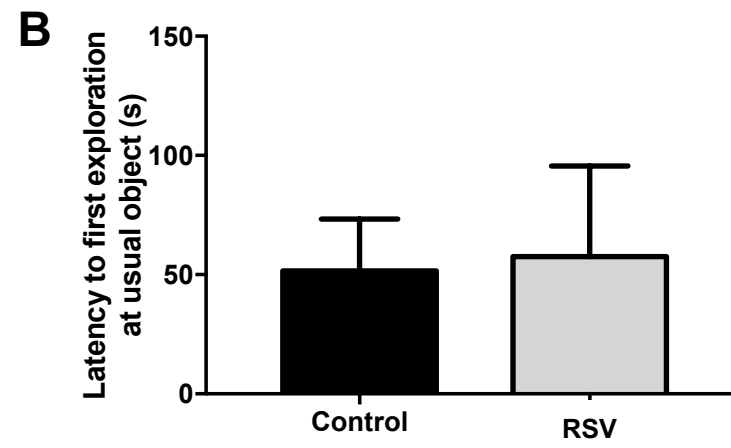
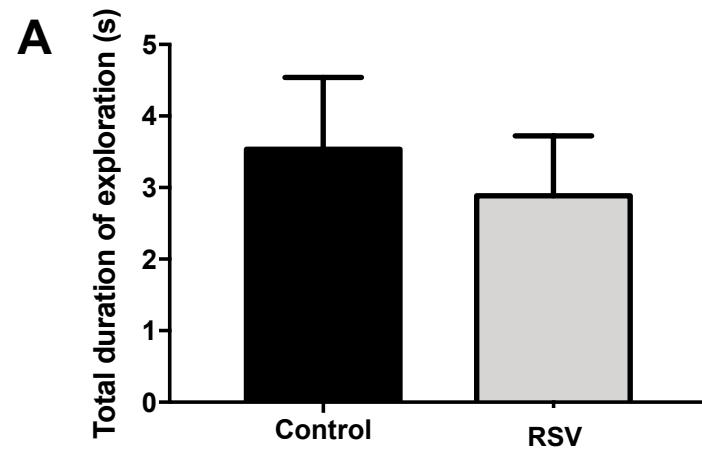
FC: Fold change; Abs FC: absolute value of Fold change

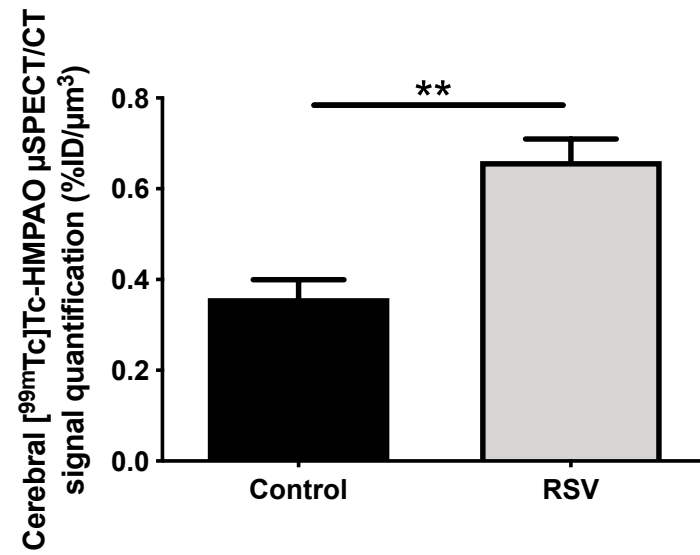
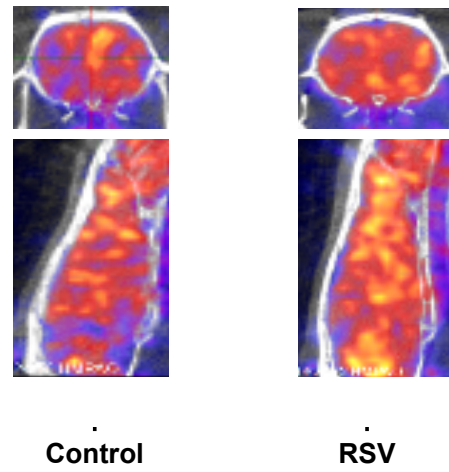
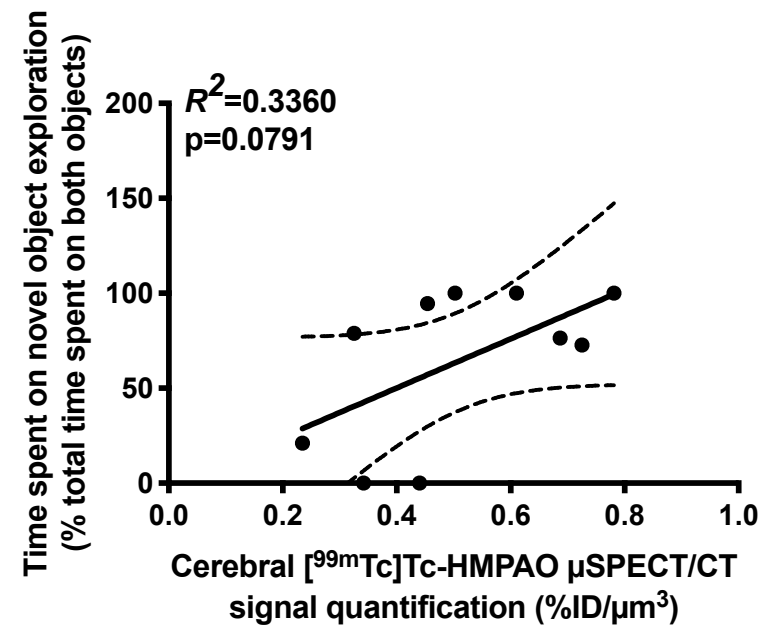
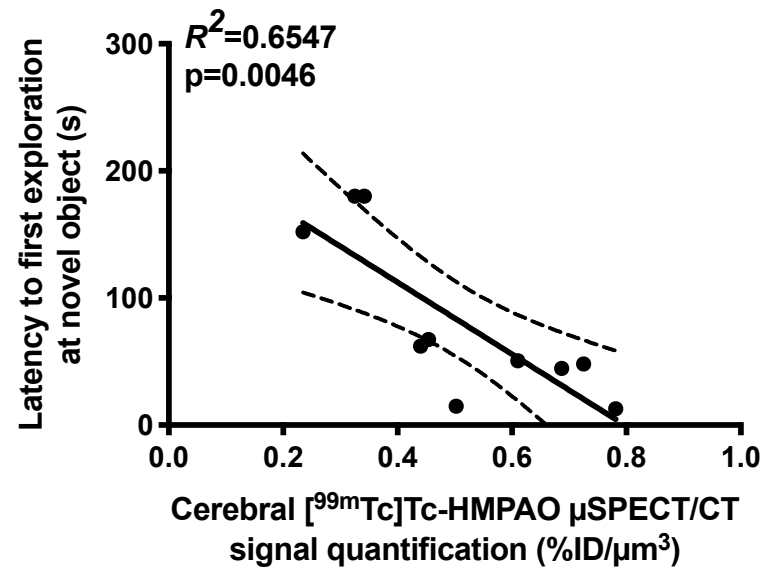
Table 2: Pathways with negative Z-score

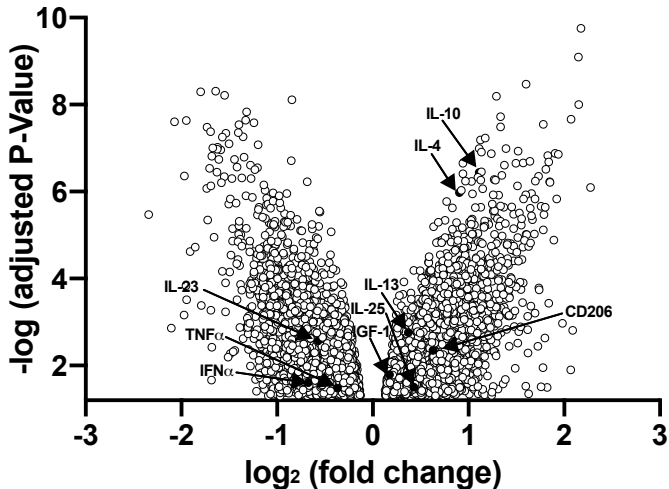
Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score
LXR/RXR Activation	5,1E00	4,63E-01	-1,826
Eicosanoid Signaling	3,42E00	4,69E-01	-0,302
MIF-mediated Glucocorticoid Regulation	3,23E00	5,6E-01	-1,387
PPAR α /RXR α Activation	2,83E00	3,51E-01	-1,257
NF- κ B Signaling	2,45E00	3,4E-01	-0,700
VDR/RXR Activation	2,33E00	3,86E-01	-0,277
MIF Regulation of Innate Immunity	2,25E00	4,55E-01	-1,807
Type I Diabetes Mellitus Signaling	2,09E00	3,6E-01	-1,460
Dendritic Cell Maturation	2,05E00	3,36E-01	-0,649
TNFR2 Signaling	1,95E00	4,62E-01	-1,732
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	1,5E00	3,85E-01	-0,447
IL-6 Signaling	1,45E00	3,19E-01	-0,333
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1,34E00	3,03E-01	-1,508
Death Receptor Signaling	1,3E00	3,29E-01	-1,400

Table 3. Pathways with positive Z-score

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score
PPAR Signaling	2,97E00	0,4	1,414
LPS/IL-1 Mediated Inhibition of RXR Function	2,29E00	3,33E-01	0,186
TWEAK Signaling	1,81E00	4,33E-01	0,832
Lymphotoxin β Receptor Signaling	1,6E00	3,62E-01	0,243
Antioxidant Action of Vitamin C	1,58E00	3,47E-01	2,294
TREM1 Signaling	1,42E00	3,6E-01	1,414
iNOS Signaling	1,4E00	3,75E-01	1,000
OX40 Signaling Pathway	1,34E00	3,82E-01	1,265



A**B****C**





5-month *per os*
1.25 mg/day
resveratrol
treatment in
senescent rats

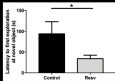


**oxidation &
inflammatory
pathways**



Control Resv

**cerebral
blood flow**



**neurocognitive
performance**