

Original Article

Myo-Inositol Dampens Apathetic Symptoms in Aged Mice and Is Associated with Sex-Specific Synapse Remodelling

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ABSTRACT: Late-life depression is associated with reduced treatment efficacy and age-related impairments in synaptic plasticity and stress responsiveness. Myo-inositol, an endogenous regulator of phosphoinositide signalling, has been proposed as candidate treatment for mood disorders, but its effects in age-related mood disorders remain unclear. Here, we investigated the impact of chronic myo-inositol supplementation on depressive behaviour and synaptic markers in aged male and female mice. Aged mice displayed reduced self-care behaviour in the sucrose splash test compared to young adults. Myo-inositol supplementation restored grooming behaviour in both sexes, an effect maintained following exposure to unpredictable chronic mild stress and independent of locomotor activity. Molecular analyses revealed sex-specific synaptic adaptations, with myo-inositol enhancing postsynaptic density protein 95 (PSD-95) levels in aged males, while increasing phosphorylation of the AMPA receptor subunit GluA1 at serine 845 and TARP expression in females. These findings demonstrate that myo-inositol dampens age-related apathetic alterations and is associated with sex-specific synaptic mechanisms, supporting its potential as a therapeutic strategy for late-life depression.

Keywords: depression, aging, myo-inositol, synaptic proteins, anhedonia.

INTRODUCTION

Late-life depression (LLD) is characterized by poor treatment response and higher relapse rates compared to depression in younger populations, highlighting the need for therapeutic strategies that specifically address age-related neurobiological vulnerabilities [1]. Indeed, aging is accompanied by profound changes in brain structure and function, including alterations in synaptic plasticity, neurotransmission, and stress responsiveness, in key hubs of the limbic system strictly involved in depression symptomatology [2, 3]. At the synaptic level, aging is associated with a progressive reduction in dendritic complexity and impaired activity-dependent plasticity [4-6]. These changes can be exacerbated by chronic stress, a major risk factor for depression, which further disrupts glutamatergic signalling and postsynaptic integrity in corticolimbic regions implicated in mood regulation [7,

8]. Consistent with these results, studies on both experimental models and human postmortem brains have reported region-specific alterations in the expression of synaptic proteins and altered glutamate receptor trafficking in depressive states, supporting the hypothesis that depression reflects, at least in part, a disorder of synaptic dysfunction rather than solely neurotransmitter imbalance [9, 10].

Key components of excitatory synapses are particularly vulnerable to both aging and stress [9, 10]. For example, postsynaptic density protein 95 (PSD-95) is the major scaffolding protein that stabilizes glutamatergic synapses and anchors AMPA and NMDA receptors at the postsynaptic membrane [11]. Reduced PSD-95 expression has been associated with synapse loss, impaired synaptic transmission, and depressive-like phenotypes in animal models [12]. In parallel, functional modulation of AMPA receptors, as well as AMPA

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subunits expression, plays a critical role in experience-dependent plasticity, stress-vulnerability and antidepressant responses [13–15]. Dysregulation of physiological mechanisms regulating synaptic structure is increasingly recognized as a convergent pathway linking aging, stress, and mood disorders [16]. Biological sex further modulates both the risk and the neurobiology of depression. For instance, women exhibit a higher lifetime prevalence of major depressive disorder (MDD), and sex differences have been documented in stress reactivity, synaptic plasticity, and intracellular signalling pathways [17, 18].

Myo-inositol is a naturally occurring carbocyclic polyol that plays a central role in phosphoinositide signalling, membrane dynamics, and intracellular second messenger pathways. In the brain, myo-inositol is abundant in neurons and glia and contributes to processes including calcium signalling, receptor trafficking, and synaptic function [19]. Alterations in inositol metabolism have been reported in mood disorders, and myo-inositol has shown antidepressant-like effects in both preclinical and clinical studies, although its mechanisms of action remain incompletely defined [20, 21]. However, whether myo-inositol can ameliorate depressive symptomatology in aged subjects, and whether such effects is associated with synaptic mechanisms in a sex-dependent manner, remains largely unexplored.

In the present study, we investigated the effects of dietary myo-inositol on depressive behaviour and synaptic protein expression in male and female mice aged between 18 and 20 months old. Using the sucrose splash test (SST), forced swim test (FST) and elevated plus maze (EPM) as measurements of anhedonia, apathetic, stress coping and anxious/risk-assessment responses, we evaluated functional recovery in conjunction with molecular markers of synaptic structure and function. We hypothesized that myo-inositol would promote behavioural recovery in aged mice and sex-specific restoration of synaptic markers expression. Our findings reveal potential synapse associated mechanisms which may be involved in the therapeutic effects of myo-inositol on age- and stress-related apathetic phenotype.

MATERIALS AND METHODS

Animals

Male and female wild-type C57Bl/6J mice (3 months and 18–20 months old) obtained from the Animal Facility of the Catholic University were used for this study. Animals were housed under standard laboratory conditions with a 12 h light/dark cycle, with constant humidity (60–75%), controlled room temperature (RT, 20–22 °C) and food and water provided *ad libitum*.

Ethics

All experiments and animal procedures were approved by the Catholic University Ethics Committee and were in accordance with the guidelines of the Italian Ministry of Health (Legislative Decree No. 116/1992) and European Union legislations on animal procedures (Directive No. 86/609/EEC), and were approved by the Italian Ministry of Health, procedure number 470/2024-PR.

Stress paradigm

A modified version of the unpredictable chronic mild stress (UCMS) paradigm, adapted from previous studies [22], was used. Mice were exposed to a randomized sequence of mild stressors administered daily for two weeks. The stress protocol included: (1) restraint stress for 3 h, (2) food or water deprivation for 6 h, (3) tilted cage at 45° for 6 h, (4) soiled bedding for 6 h, (5) tail pinch or suspension for 3 min, and (6) cold water swimming for 5 min. Two stressors were applied per day. The UCMS lasted for the 5th and 6th week of myo-inositol exposure.

Myo-inositol treatment

Myo-inositol (Sigma-aldrich, #57570) was diluted in drinking water at 0.5% weight/volume. Considering a 4–6 mL range of water consumption per day at 0.5% w/v, for mice weighing on average 30 grams, corresponds to a dose between 0.6 to 1 g/Kg, in line with human doses used in clinical contexts after applying conversions for metabolic differences between species [20, 23]. Fresh inositol was changed every 3 days, to avoid fungal contamination. For experiments shown in figure 2 and 3, myo-inositol treatment was started at 18/20 months of age and was kept for 4 and 6 weeks.

Behavioural analysis

Behavioural assessments were conducted as previously reported [22]. Briefly, behavioural analyses were performed from 9:00 a.m. to 2:00 p.m. and data were collected and blindly analysed using the behavioural tracking system ANY-MazeTM (Stoelting Co., Wood Dale, IL, USA).

Sucrose splash test

Anhedonia and apathy were evaluated using the sucrose splash test (SST). Each mouse was sprayed on its dorsal coat with seven squirts of a 10% sucrose solution and immediately placed in a transparent plexiglass cylinder (12 cm diameter, 20 cm height). Self-grooming behaviour

was recorded for 5 min. Reduction in time spent grooming is indicative of apathetic behaviour and anhedonia.

Forced swim test

Stress-coping response was assessed by the forced swim test (FST). Mice were placed in a transparent baker (12 cm diameter, 16 cm height; Vitlab GmbH) containing water at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ to a depth of 10 cm, leaving a 6 cm margin from the rim. Each session lasted 6 min, and total immobility time was recorded during the last 4 min. Water was changed every 10 animals to ensure stable temperature conditions.

Elevated plus maze

Anxiety was assessed using the elevated plus maze (EPM, Ugo Basile, VA, Italy). The apparatus consisted of two opposing open arms (30×5 cm) and two equal-sized closed arms ($30 \times 5 \times 15$ cm) opposite each other, elevated 50 cm above the floor. Each mouse was placed in the central square (5×5 cm) facing an open arm and allowed to explore the maze for 5 min. The total distance travelled, time spent in the open and closed arms, as well as head-dipping behaviour, were recorded. Head dipping is an exploratory behaviour associated with curiosity and risk assessment in novel environments, in which animals lower their heads beyond the open arms of the maze. The apparatus was cleaned with a 70% ethanol solution after each session.

Western blot analyses

At the end of the behavioural tests, the animals were sacrificed via cervical dislocation for brain tissues collection. After decapitation, the brain was rapidly removed and then dissected on ice to isolate the prefrontal cortex followed by lysis as previously described [24-27]. Specifically, tissues were lysed in ice-cold lysis buffer (NaCl 150 mM, Tris-HCl 50 mM pH 8, and EDTA 2 mM) containing 1% Triton X-100, 0.5% SDS, 1× protease inhibitor cocktail (Sigma-Aldrich), 1 mM sodium orthovanadate (Sigma-Aldrich), and 1 mM sodium fluoride (Sigma-Aldrich). Lysates were then sonicated for 5 min using a Bioruptor Standard Waterbath Sonicator (Diagenode, Liège, Belgium) and centrifuged at 13,000 rcf for 13 min at 4°C . Equal amounts of protein were diluted in a 6× Laemmli buffer, boiled, and resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Equal amounts of protein were loaded onto 8% Tris-glycine polyacrylamide gels (Bio-Rad) for electrophoretic separation. Precision Plus Protein™ Dual Color Standards (Bio-Rad) were used as molecular mass markers. Proteins were transferred onto polyvinylidene

fluoride membranes (Millipore) and stained with Ponceau S solution to verify the uniformity of protein loading. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at RT and then incubated overnight at 4°C with the following primary antibodies: anti-PSD-95 (1:1000, ab18258 Abcam), anti-phospho-AMPA Receptor 1 (GluA1) (Ser845) (1:1000, #8084 Cell Signaling), anti-GLUA1 (1:1000, MAB2263 Millipore), anti-NMDAR2A (1:1000, 07-632 Millipore), anti-NMDAR2B (1:1000, ab93610 Abcam), Stargazin/TARP- γ 2/8 (1:1000, Ab9876 Millipore), β -Actin (1:1000, ab8227 Abcam). After three 10-min washes in TBST, the membranes were incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology #7074S, 1:3000). The membranes were washed again, and the immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Thermo Fisher Scientific) and imaged using a ChemiDoc Imaging System (Bio-Rad). Protein expression was evaluated and documented by UVItec Cambridge Alliance. Images shown were cropped for presentation with no manipulations. Full uncropped blots are reported in supplementary material.

Statistical analysis

Sample sizes for behavioural analyses were chosen with adequate statistical power (0.8) using G*Power software and according to results of prior pilot data sets or studies, including our own, using similar methods or paradigms. For biochemical analyses, sample size was selected based on previously published papers. Data was analysed using GraphPad Prism software. Data are expressed as mean $\pm$ standard error of the mean (SEM), with significance set at $p < 0.05$. Data was first tested for equal variance and normality (Shapiro-Wilk test). Behavioural results were analysed using a two-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test. Western immunoblotting data were analysed using one-way ANOVA, followed by a Tukey's post hoc test or non-parametric equivalent analyses. Animals were randomly allocated to experimental groups prior to treatment initiation using a simple randomization procedure that allowed distribution of age matched animals across conditions (age range: 18-20). Allocation concealment was ensured by assigning coded identifiers to each animal before group assignment, such that the investigator administering treatment was unaware of group allocation. Investigators were blinded during behavioural testing, tissue collection and Western blot quantification.

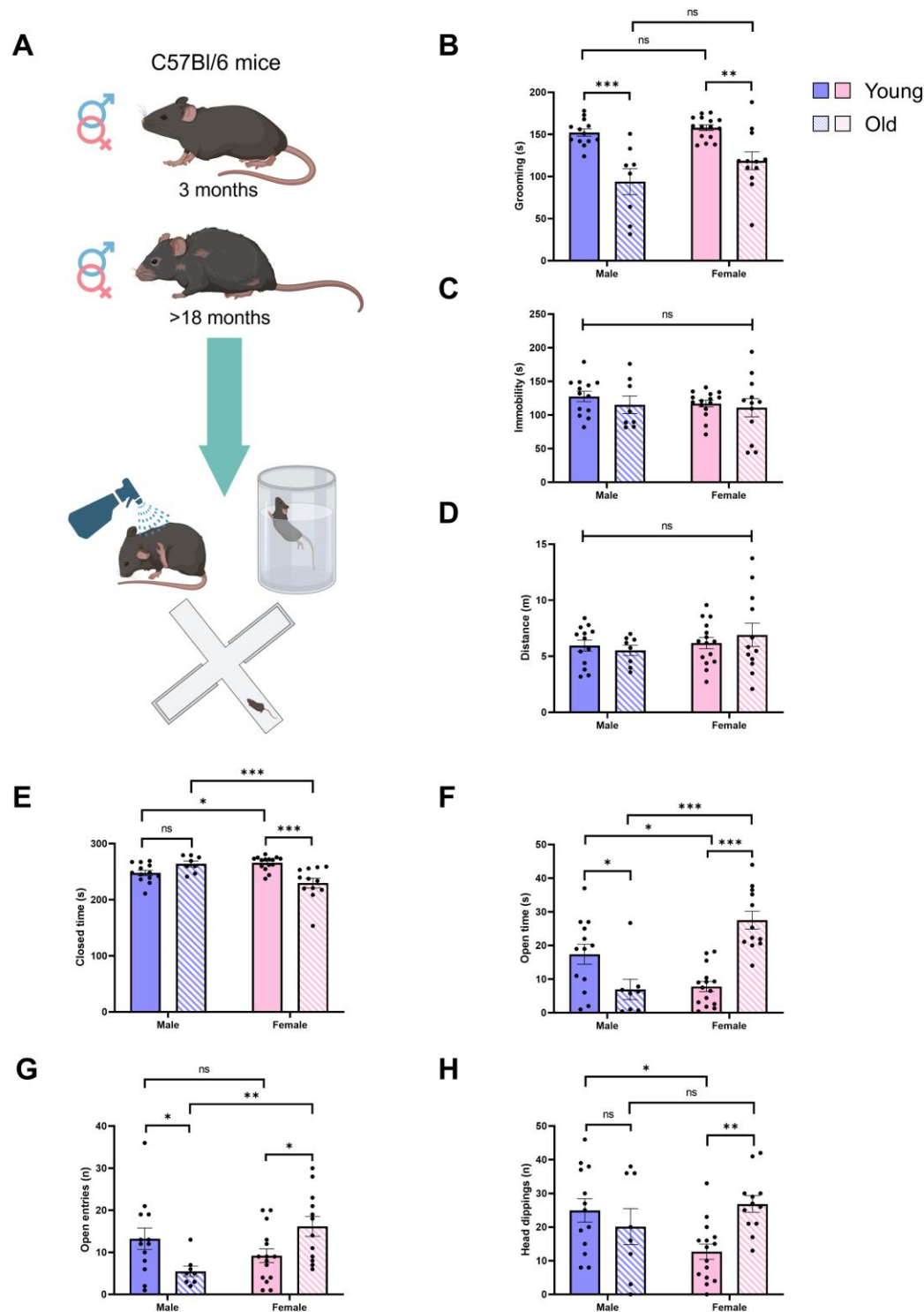


Figure 1. Age- and sex-dependent behavioural characterization of C57Bl/6 mice. (A) Experimental design: young (3 months) and aged (18-20 months) male and female C57Bl/6 mice were subjected to behavioural testing. SST (B) and FST (C) results in male and female mice. (D) Total distance travelled in EPM test. (E-H) From the left to the right: anxiety behaviour assessed by the closed and open arms time, number of open arm entries and head-dipping events in the EPM in male and female mice. Sample size: young males n=13; old males n=8; young females n=15; old females n=12. Data are presented as mean $\pm$ SEM. Each dot represents an individual animal. Statistics by two-way ANOVA, Bonferroni post hoc; *p<0.05, **p<0.01, ***p<0.001; ns: not significant.

RESULTS

Aging is characterized by sex-specific alterations in anxious and depressive behaviours

We first characterized the extent of depressive and anxious behaviour in both male and female mice of 18-20 months old, comparing them to sex-matched young adults of 3 months of age. To this aim, animals were subjected to SST, FST and EPM, to assess apathetic behaviours, anhedonia, stress coping responses and anxious/exploratory behaviours (Fig. 1A). In the SST, both male and female aged animals showed a significant decrease in time spent self-grooming compared to young mice (main effect of age, $F_{(1, 44)}=35.85$, $p<0.001$, Cohen's $f=0.90$; males: young vs. old $152.2\pm4.3s$ vs. $93.9\pm15.4s$, $p<0.001$; females: young vs. old $157.9\pm3.4s$ vs. $118.7\pm10.5s$, $p=0.001$; two-way ANOVA, Bonferroni post hoc; Fig. 1B). In the FST, no differences were observed between young and old mice, for both sexes ($p>0.05$ for both comparisons; two-way ANOVA; Fig. 1C). In the EPM, we did not observe a reduction in distance covered in both sexes ($p>0.05$; two-way ANOVA, Bonferroni post hoc; Fig. 1D). Regarding time spent in closed arms of the EPM, we did not observe an effect of sex ($F_{(1, 44)}=1.984$, $p>0.05$, Cohen's $f=0.21$) or age ($F_{(1, 44)}=2.962$, $p>0.05$, Cohen's $f=0.25$) but a significant effect of interaction ($F_{(1, 44)}=20.37$, $p<0.001$, Cohen's $f=0.68$, Fig. 1E). Post-hoc analyses revealed no significant differences between young males and old males (males: young vs. old $247.6\pm4.6s$ vs. $263.7\pm5.1s$, $p=0.144$), reduced time for aged females compared to their young counterpart (females: young vs. old $265.5\pm3.2s$ vs. $229.6\pm8.5s$, $p<0.001$) along with sex-differences (young: males vs. females $247.6\pm4.6s$ vs. $265.5\pm3.2s$, $p=0.039$; old: males vs. females $263.7\pm5.1s$ vs. $229.6\pm8.5s$, $p=0.001$). Regarding time spent in open arms, we observed no effect of age, but a significant effect of sex ($F_{(1, 44)}=4.610$, $p=0.037$, Cohen's $f=0.31$) and interaction ($F_{(1, 44)}=34.57$, $p<0.001$, Cohen's $f=0.88$; males: young vs. old $17.4\pm3.0s$ vs. $6.9\pm3.0s$, $p=0.020$; females: young vs. old $7.8\pm1.5s$ vs. $27.6\pm2.7s$, $p<0.001$; young: males vs. females $17.4\pm3.0s$ vs. $7.8\pm1.5s$, $p=0.011$; old: males vs. females $6.9\pm3.0s$ vs. $27.6\pm2.7s$, $p<0.001$; two-way ANOVA, Bonferroni post hoc; Fig. 1F). Concerning open arms entries there was no effect of sex or age ($p>0.05$) but a main effect of interaction ($F_{(1, 44)}=11.19$, $p=0.002$, Cohen's $f=0.50$; males: young vs. old 13.2 ± 2.6 vs. 5.5 ± 1.2 , $p=0.049$; females: young vs. old 9.2 ± 1.3 vs. 16.2 ± 2.4 , $p=0.039$; young: males vs. females 13.2 ± 2.6 vs. 9.2 ± 1.3 , $p=0.316$; old: males vs. females 5.5 ± 1.2 vs. 16.2 ± 2.4 , $p=0.006$; two-way ANOVA, Bonferroni post hoc; Fig. 1G). Finally, analysis of head dipping behaviour revealed a main effect of interaction (effect of interaction, $F_{(1, 44)}=8.34$, $p=0.006$,

Cohen's $f=0.43$; males: young vs. old 24.9 ± 3.5 vs. 20.1 ± 5.4 , $p=0.680$; females: young vs. old 12.7 ± 2.3 vs. 26.8 ± 2.5 , $p=0.004$; young: males vs. females 24.9 ± 3.5 vs. 12.7 ± 2.3 , $p=0.011$; old: males vs. females 20.1 ± 5.4 vs. 26.8 ± 2.5 , $p=0.382$; two-way ANOVA, Bonferroni post hoc; Fig. 1H). These data indicate that both male and female aged animals show disturbances of apathetic and self-care behaviour, sex specific patterns in anxiety and risk-assessment behaviour but no alterations in stress-coping responses.

Myo-inositol improves apathetic alterations in aged mice

Recent evidence indicates that alterations in inositol metabolism could be associated with depressive disorders and that, in some cases, administration of exogenous myo-inositol could restore psychological deficits [28, 29]. Therefore, we sought to determine whether myo-inositol supplementation could be employed for the treatment of age-dependent depressive alterations. To this end, aged animals were treated for 4 weeks with myo-inositol supplementation in drinking water (Fig. 2A). In the SST, we observed a significant effect of treatment (main effect of treatment, $F_{(1, 64)}=16.62$, $p<0.001$, Cohen's $f=0.50$; males: vehicle vs. myo-inositol $68.5\pm11.7s$ vs. $112.5\pm9.0s$, $p=0.004$; females: vehicle vs. myo-inositol $113.2\pm8.2s$ vs. $143.8\pm8.0s$, $p=0.032$; two-way ANOVA, Bonferroni post hoc; Fig. 2B) and of sex (main effect of sex, $F_{(1, 64)}=17.18$, $p<0.001$, Cohen's $f=0.51$; vehicle: males vs. females $68.5\pm11.7s$ vs. $113.2\pm8.2s$, $p=0.004$; myo-inositol: males vs. females $112.5\pm9.0s$ vs. $143.8\pm8.0s$, $p=0.032$; two-way ANOVA, Bonferroni post hoc; Fig. 2B) but no effects of interaction ($p>0.05$). In the FST, two-way ANOVA did not reveal an effect of sex or treatment, but a main effect of interaction (effect of interaction, $F_{(1, 64)}=4.39$, $p=0.040$, Cohen's $f=0.26$; males: vehicle vs. myo-inositol $110.2\pm10.7s$ vs. $116.1\pm7.5s$, $p>0.05$; females: vehicle vs. myo-inositol $143.3\pm7.3s$ vs. $109.0\pm12.1s$, $p=0.020$; vehicle: males vs. females $110.2\pm10.7s$ vs. $143.3\pm7.3s$, $p=0.040$; myo-inositol: males vs. females $116.1\pm7.5s$ vs. $109.0\pm12.1s$, $p>0.05$; two-way ANOVA, Bonferroni post hoc; Fig. 2C). Concerning the EPM, we observed for closed arms time an effect of sex and interaction (Closed: main effect of sex, $F_{(1, 64)}=6.43$, $p=0.014$, Cohen's $f=0.31$; effect of interaction, $F_{(1, 64)}=8.59$, $p=0.005$, Cohen's $f=0.35$; males: vehicle vs. myo-inositol $265.8\pm3.2s$ vs. $260.1\pm2.7s$, $p=0.785$; females: vehicle vs. myo-inositol $241.4\pm6.5s$ vs. $261.9\pm3.1s$, $p=0.002$; vehicle: males vs. females $265.8\pm3.2s$ vs. $241.4\pm6.5s$, $p<0.001$; myo-inositol: males vs. females $260.1\pm2.7s$ vs. $261.9\pm3.1s$, $p>0.05$; two-way ANOVA, Bonferroni post hoc; Fig. 2D) while for open arms time we reported an effect of sex, of treatment and

of interaction as well (Sex, $F_{(1,64)}=8.44$, $p=0.005$, Cohen's $f=0.36$; Treatment, $F_{(1,64)}=4.12$, $p=0.047$, Cohen's $f=0.25$; Interaction, $F_{(1,64)}=5.90$, $p=0.018$, Cohen's $f=0.30$; males: vehicle vs. myo-inositol $9.1\pm2.1s$ vs. $10.3\pm1.8s$, $p>0.05$; females: vehicle vs. myo-inositol $24.5\pm4.4s$ vs. $11.7\pm1.5s$,

$p=0.003$; vehicle: males vs. females $9.1\pm2.1s$ vs. $24.5\pm4.4s$, $p<0.001$; myo-inositol: males vs. females $10.3\pm1.8s$ vs. $11.7\pm1.5s$, $p>0.05$; two-way ANOVA, Bonferroni post hoc; Fig. 2E).

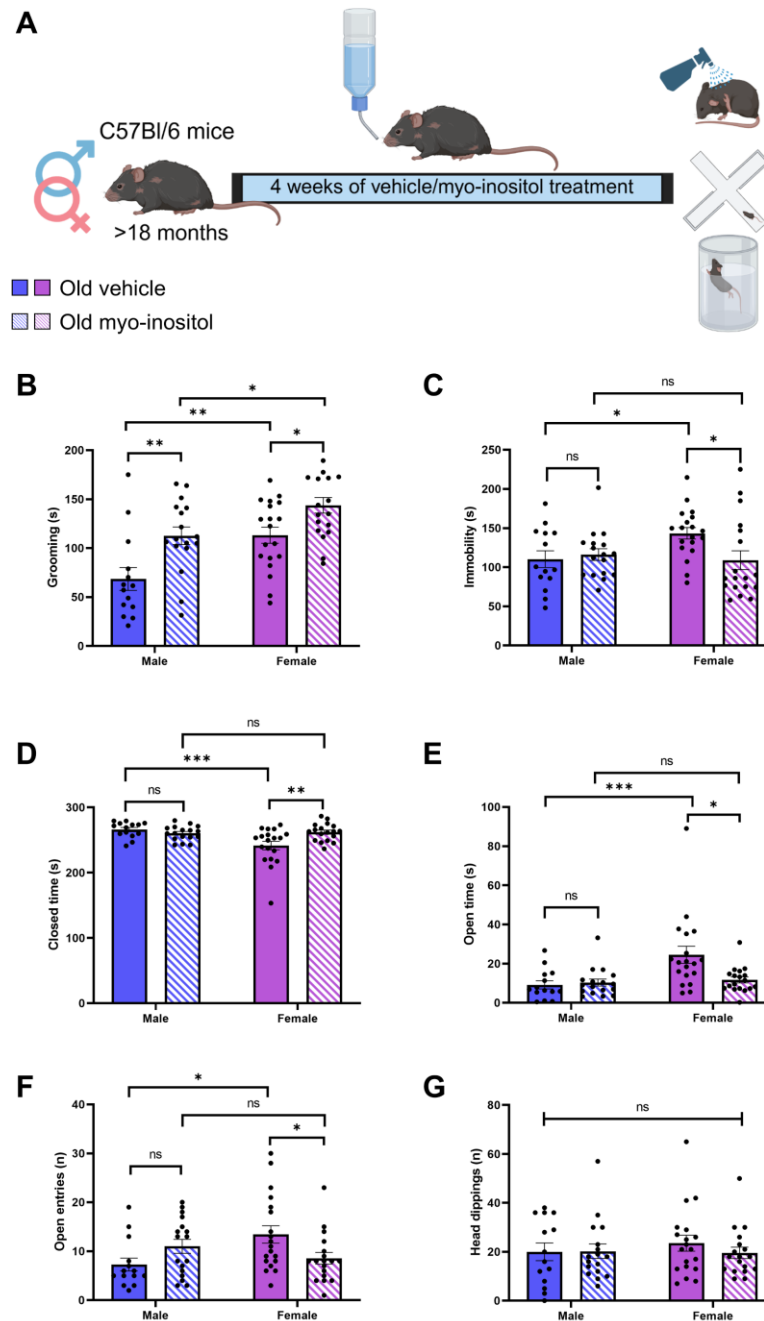


Figure 2. Effects of 4 weeks of myo-inositol treatment on behavioural outcomes in aged C57Bl/6 mice. (A) Experimental design: aged (>18 months) C57Bl/6 mice were treated for 4 weeks with vehicle or myo-inositol, followed by behavioural testing. SST (**B**) and FST (**C**) results in male and female mice. (**D-G**) EPM parameters in male and female mice. Sample size: males Old vehicle n=14; males Old myo-inositol n=17; female Old vehicle n=19; female Old myo-inositol n=18. Data are presented as mean ± SEM. Each dot represents an individual animal. Statistics by two-way ANOVA, Bonferroni post hoc; *p<0.05, **p<0.01; ***p<0.001; ns: not significant.

Number of open arms entries was characterized instead by a significant effect of interaction (effect of interaction, $F_{(1,64)}=8.35$, $p=0.005$, Cohen's $f=0.36$; males: vehicle vs. myo-inositol 7.3 ± 1.3 vs. 11.0 ± 1.41 , $p=0.191$; females: vehicle vs. myo-inositol 13.4 ± 1.8 vs. 8.6 ± 1.2 , $p=0.036$; vehicle: males vs. females 7.3 ± 1.3 vs. 13.4 ± 1.8 , $p=0.011$; myo-inositol: males vs. females 11.0 ± 1.41 vs. 8.6 ± 1.2 , $p=0.478$; two-way ANOVA, Bonferroni post hoc; Fig.

2F), while no effects were observed for head-dipping behaviour ($p>0.05$ for both sexes; two-way ANOVA; Fig. 2G). Finally, myo-inositol treatment did not change distance travelled for both sexes and treatment condition, thus suggesting that the effects that we observed were independent from locomotor activity ($p>0.05$ for both sexes; two-way ANOVA; Supplementary Fig. 1A).

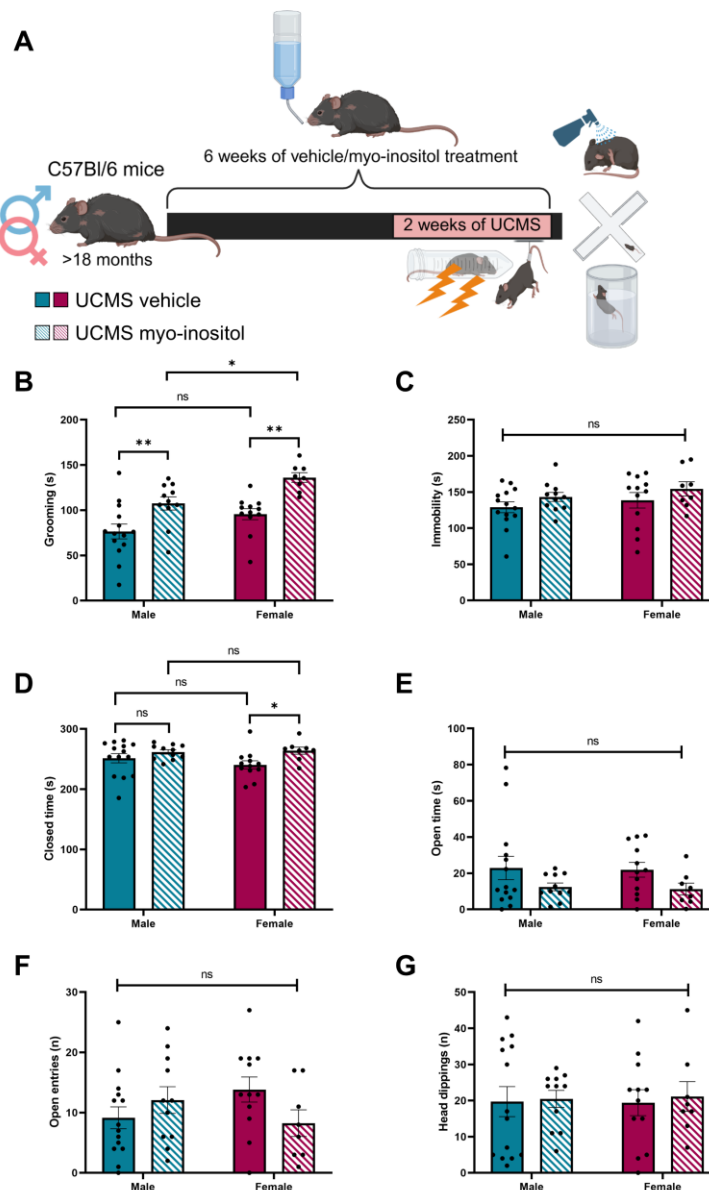


Figure 3. Effects of 6 weeks of myo-inositol treatment on behavioural outcomes in 18 m.o. stressed C57Bl/6 mice. (A) Experimental design: aged (>18 months) C57Bl/6 mice were treated with vehicle or myo-inositol for 4 weeks before and 2 weeks during UCMS protocol, followed by behavioural assessment. SST (B) and FST (C) results in male and female mice. (D-G) EPM parameters in male and female mice. Sample size: male UCMS vehicle $n=14$; male UCMS myo-inositol $n=11$; female UCMS vehicle $n=13$; female UCMS myo-inositol $n=8$. Data are presented as mean $\pm$ SEM. Each dot represents an individual animal. Statistics by two-way ANOVA, Bonferroni post hoc; * $p<0.05$, ** $p<0.01$, ns: not significant.

Of note, myo-inositol intake normalized by weight did not differ between male and female mice and myo-inositol did not impact body weight between treated and non-treated animals (See Supplementary Fig. 2A and B). Collectively, these data suggest that myo-inositol supplementation can improve mood alterations in the aged population, further showing sex-specific effects: in particular, myo-inositol treatment was able to improve SST, FST and EPM performance in females, while in males it promoted only a functional recovery in the SST.

Myo-inositol reduces vulnerability to UCMS in both aged male and female mice

We then asked whether myo-inositol treatment could also influence the vulnerability to stress exposure in aged mice. To this end, a separate cohort of either vehicle- or myo-inositol-exposed aged mice were subjected to the UCMS paradigm for the last 2 weeks of treatment, as shown in Figure 3A. In both male and female animals, myo-inositol pre-treatment resulted in lower apathetic behaviour after stress-exposure compared to vehicle-treated mice (main effect of treatment, $F_{(1, 41)}=23.06$, $p<0.001$, Cohen's $f=0.74$; males: UCMS vehicle vs. UCMS myo-inositol $76.5\pm 8.2s$ vs. $107.4\pm 7.2s$, $p=0.006$;

females: UCMS vehicle vs. UCMS myo-inositol $95.6\pm 6.1s$ vs. $136.1\pm 5.3s$, $p=0.002$; two-way ANOVA, Bonferroni post hoc; Fig. 3B). Two-way ANOVA also revealed a significant effect of sex (Sex, $F_{(1, 41)}=10.31$, $p=0.002$, Cohen's $f=0.50$; UCMS vehicle: males vs. females $76.5\pm 8.2s$ vs. $95.6\pm 6.1s$, $p=0.107$; UCMS myo-inositol: males vs. females $107.4\pm 7.2s$ vs. $136.1\pm 5.3s$, $p=0.031$; two-way ANOVA, Bonferroni post hoc; Fig. 3B). No differences were observed in the immobility time during FST for both male and female mice ($p>0.05$; two-way ANOVA; Fig. 3C). Regarding the EPM, we observed a significant effect of treatment for closed arms time (Treatment, $F_{(1, 41)}=6.33$, $p=0.016$, Cohen's $f=0.39$; males: UCMS vehicle vs. UCMS myo-inositol $251.2\pm 7.7s$ vs. $261.6\pm 3.6s$, $p=0.500$; females: UCMS vehicle vs. UCMS myo-inositol $240.2\pm 6.8s$ vs. $263.8\pm 5.9s$, $p=0.050$; UCMS vehicle: males vs. females $251.2\pm 7.7s$ vs. $240.2\pm 6.8s$, $p=0.430$; UCMS myo-inositol: males vs. females $261.6\pm 3.6s$ vs. $263.8\pm 5.9s$, $p>0.05$; two-way ANOVA, Bonferroni post hoc; Fig. 3D). However, no differences were recorded for open arms time, open arms entries and head-dipping behaviour ($p>0.05$; two-way ANOVA, Bonferroni post hoc; Fig. 3E to G). These results indicate that myo-inositol treatment mainly affects apathetic behaviours in aged animals subjected to stress.

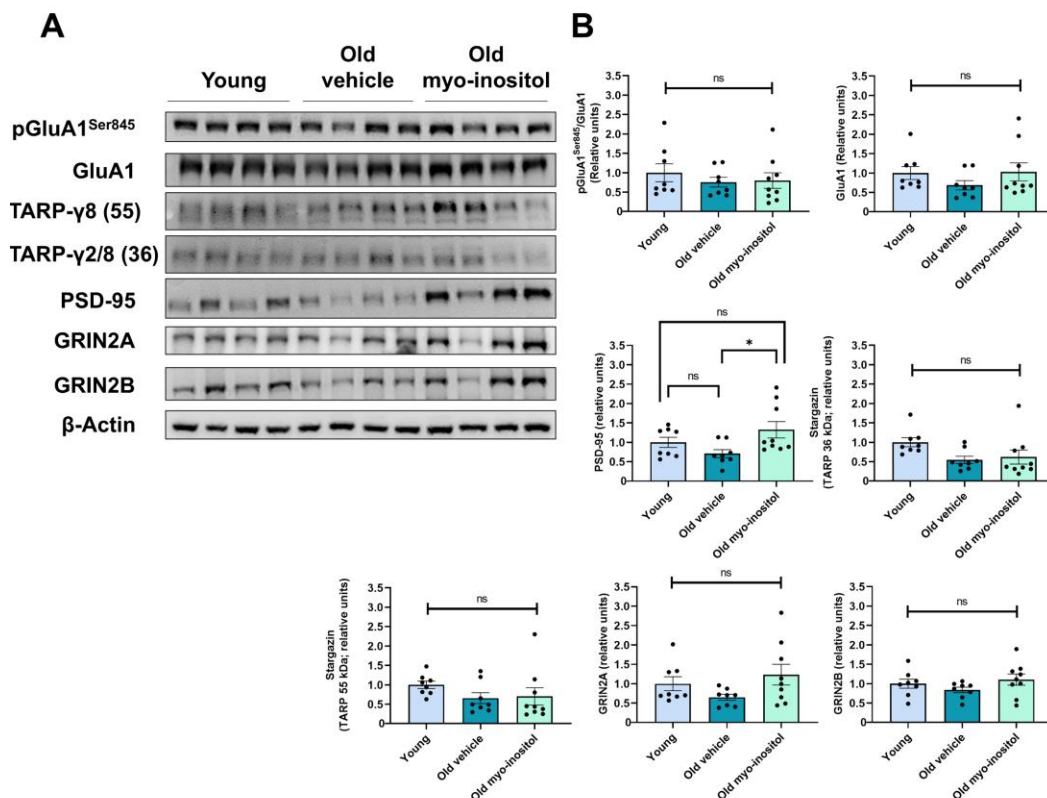


Figure 4. Myo-inositol treatment increases PSD-95 expression in the mPFC of aged male mice. (A) Representative immunoblots showing the levels of GluA1 phosphorylation at Ser845, total GluA1, Stargazin (TARP

55 kDa and TARP 36 kDa), PSD-95, GRIN2A and GRIN2B between young and aged male mice treated with either vehicle or myo-inositol. (B) Bar graphs showing quantification of synaptic protein expression in different experimental groups. We did not observe any significant variation between young and aged mice. On the other hand, myo-inositol treatment was able to induce a significant increase in PSD-95 expression in aged males. Each group comprised $n=8-9$. Data are presented as mean $\pm$ SEM. Each dot represents an individual animal. Statistics by one-way ANOVA followed by post-hoc analysis; * $p<0.05$; ns: not significant.

Myo-inositol induces sex-specific changes of synaptic plasticity-related protein expression in the prefrontal cortex of aged mice

Finally, we wanted to evaluate whether inositol supplementation could impact synaptic remodelling in aged mice. Indeed, recent evidence indicates that inositol promotes synaptogenesis in the infant cortex and in human cortical neurons [30]. To this end, we investigated whether myo-inositol impacted the expression of synaptic markers in the medial prefrontal cortex (mPFC) of aged mice. In male mice, despite observing a decreasing trend, no significant variations in the level of multiple synaptic markers, namely GluA1 phosphorylation at Ser845, total GluA1, Stargazin (TARP at 36 and 55 kDa), PSD-95, GRIN2A and GRIN2B, from young to aged condition were reported (Fig. 4A and B). However, myo-inositol supplementation was able to increase the levels of PSD-95 in the mPFC of aged mice, which was significantly higher compared to old mice treated with vehicle ($p=0.04$, one-way ANOVA; Old vehicle vs. Old myo-inositol:

0.70 ± 0.10 vs. 1.32 ± 0.21 , $p=0.032$, one-way ANOVA, Tukey's post-hoc, Fig. 4A and B, see Supplementary Fig. 3A for full uncropped blots). Regarding female mice, a decreasing trend in young vs. old vehicle was observed, although not significant. Myo-inositol treatment did not impact significantly PSD-95 expression ($p>0.05$; one-way ANOVA, Tukey's post-hoc, Fig. 5B), but instead increased the levels of GluA1 phosphorylation at serine 845 ($p=0.024$, Kruskal-Wallis ANOVA; Old vehicle vs. Old myo-inositol: 0.92 ± 0.11 vs. 1.62 ± 0.26 , $p=0.019$; Dunn's multiple comparison, Fig. 5A and B), a post-translational modification involved in AMPAR trafficking, as well as the level of Stargazin/TARP γ -8 weighing 55 kDa ($p=0.021$, one-way ANOVA; Old vehicle vs. Old myo-inositol: 0.41 ± 0.07 vs. 1.30 ± 0.19 , $p=0.019$; Tukey's post-hoc; Fig. 5A and B), which is associated with functional TARP γ -8 glycosylation [31]. Collectively, these data indicate that myo-inositol treatment in aged mice is associated with enhanced expression of sex-specific synaptic proteins.

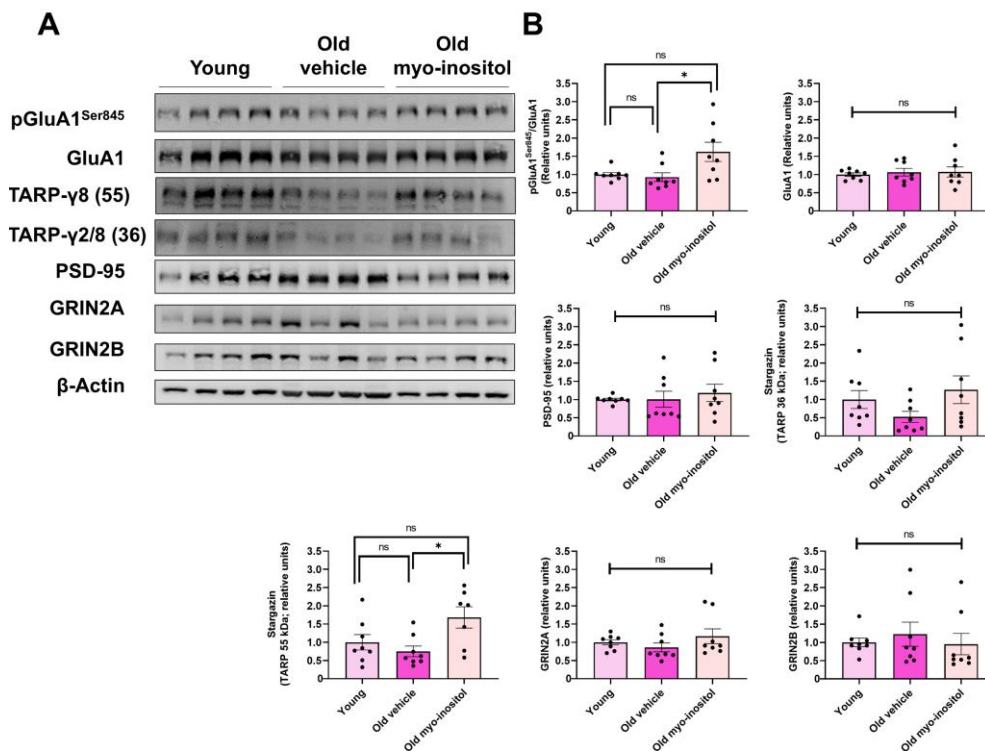


Figure 5. Myo-inositol treatment increases GluA1 phosphorylation at serine 845 and Stargazin expression in the mPFC of aged female mice. (A) Immunoblots showing changes in the levels of GluA1

phosphorylation at Ser845, total GluA1, Stargazin (TARP 55 kDa and TARP 36 kDa), PSD-95, GRIN2A and GRIN2B between young and aged female mice treated with either vehicle or myo-inositol. **(B)** Bar graphs showing quantification of synaptic protein expression in different experimental groups. No differences were observed between young and aged females, although Stargazin levels almost reached significance. On the other hand, myo-inositol significantly increased the levels of GluA1 phosphorylation at Ser845 and Stargazin (TARP 55kDa) expression. Each group comprised n=8-9. Data are presented as mean $\pm$ SEM. Each dot represents an individual animal. Statistics by one-way or Kruskal-Wallis ANOVA followed by post-hoc analysis; * $p < 0.05$, ns: not significant.

DISCUSSION

Aging is associated with a decline in multiple aspects of cognitive and psychological functions, which can also lead to higher vulnerability to different diseases, such as neurodegenerative disorders [32] and/or some kinds of neuropsychiatric disorders, such as LLD. Indeed, during aging, multiple systems in the brain can reduce their activity and their flexibility/adaptability to exogenous threats, leading to the development of depression-related characteristic alterations. Synaptic aging involves changes in the number of synapses, in the type and distribution of dendritic spines, a shift in the excitatory/inhibitory balance, circuit rearrangement, which can happen in both intra- and inter-regions [33-35]. Again, proliferative events such as adult neurogenesis, which has been often associated with antidepressant efficacy as well as depression vulnerability, is heavily reduced in aging [36]. Alterations in neuroinflammatory or metabolic signalling are also common in aging and contribute to synaptic dysfunctions [37, 38]. Overall, multiple phenomena underlie the disruption of cognitive and psychological regulations observed in aged individuals.

The SST measures both anhedonia and apathetic components of behaviour, as self-care is often a reduced function in aged individuals [39]. Although no other work has assessed SST performances in aged mice, our results are in line with those of others reporting anhedonia in aged mice assessed, for instance, by the sucrose preference test [40]. Importantly, SST may be particularly suited to detect mood-related alterations in aged populations, as it captures deficits in motivational drive and self-care behaviours that are highly relevant to LLD, although it does not fully recapitulate nor it represent the complex symptomatology of LLD. In elderly patients, reduced self-care, apathy, and psychomotor slowing often represent core symptoms that are poorly responsive to conventional antidepressant treatments and may drive and further aggravate other pathological conditions associated with aging. Thus, the ability of myo-inositol to restore grooming behaviour in aged mice may reflect an improvement in motivational and goal-directed processes rather than general changes in activity or stress coping. Regarding the FST, we failed to observe an effect in all conditions tested in aged mice, except for myo-inositol

treated female mice without stress. However, the two phenomena could be strictly connected: indeed, among the many parameters affecting the FST, it has been shown that coat state can deeply influence FST results, as air bubbles trapped within the animal fur can help it float [41]. Reduced self-care could therefore lead to altered coat state, which could have hindered the performance of aged mice, forcing them to swim for most of the time. Concerning the EPM, aged male mice showed increased anxious behaviour compared to younger ones while, in females, we observed a paradoxical effect, with aged mice showing increasing entries and exploration of open arms. To date, the impact of aging on EPM performances is still debated, as different reports have shown that aging can increase exploration into open arms, an effect that could be associated with altered risk-assessment behaviours and impulsivity [42-45]. Indeed, other reports have shown that in the EPM the benzodiazepine administration leads to increased exploratory activity, which represents a behaviour that can be attributed not only to reduced anxiety, but also to increase in risk-taking behaviour and impulsivity [46].

Multiple lines of evidence point toward myo-inositol metabolism in the regulation of stress responses, as well as to the possibility to employ its supplementation in humans for the treatment of anxious/depressive symptoms. Preclinical studies in younger rodent models of depression have shown that myo-inositol can improve mood-related outcomes across several paradigms [47], although systematic investigation remains limited and the only mechanistic effect reported is related to modulation of 5-HT₂ receptors [48]. The first evidence associated with myo-inositol alterations in depressive disorders in humans was reported in 1978, when reduced levels were reported in the cerebrospinal fluid of patients with mood disorders. Since then, multiple studies reported that low levels of myo-inositol in the prefrontal cortex of subjects with bipolar disorders were associated with higher severity of depressive symptoms, while increased levels instead were associated with manic symptoms [20, 28]. However, clinical evidence shows mixed results: in some cases, myo-inositol supplementation was able to reduce the burden of depressive symptoms, while, in other studies, it did not differ from placebo, nor it was able to improve the efficacy of standard monotherapies [20]. Of note, convincing results indicate instead, that myo-

inositol administration can significantly alleviate depressive symptoms in premenstrual dysphoric disorder patients, thus suggesting that myo-inositol could be helpful in treating depressive alterations under specific circumstances, such as in LLD [49]. In this context, our study demonstrates for the first time that myo-inositol treatment improves the apathetic phenotype in a model of LLD [40]. Accordingly, in our experimental model myo-inositol administration was able to ameliorate apathetic alterations in both male and female aged mice, under both baseline conditions as well as in the presence of a stressor. Indeed, female mice also showed a reduction in immobility in the FST when exposed to myo-inositol, although this effect was abolished in stressful conditions; furthermore, the paradoxical effect we had observed in the comparison young females vs. old females was reduced by myo-inositol treatment. This effect may be explained by a restoration of mPFC functionality, which, by regulating decision-making, can restore correct risk-assessment and risk-taking behaviour. For example, recent evidence indicates that, in rats, knockdown of myo-inositol synthesizing enzyme within the infralimbic region of the prefrontal cortex, as well as low level of myo-inositol in the same region in wild-type animals, was associated with greater impulsivity and lack of self-control [50]. In aged males, myo-inositol treatment was able to halt SST deficits, but not other parameters. In aged populations, where stress exposure often exacerbates symptom severity and reduces treatment efficacy, interventions capable of preserving functional outcomes under stress are of relevance, therefore the ability of myo-inositol to sustain behavioural improvements despite additional stressors further supports its potential utility in LLD.

A key finding of the present study is that myo-inositol supplementation induced the expression of distinct synaptic proteins. In males, behavioural recovery was associated with restoration of PSD-95 levels, a critical scaffolding protein required for synaptic stability and maintenance [12]. In contrast, in females, myo-inositol selectively increased phosphorylation of the AMPA receptor subunit GluA1 at serine 845, a modification known to enhance receptor trafficking and synaptic efficacy [11], as well as the expression of Stargazin, in particular TARP γ -8, which is an AMPA auxiliary protein helping receptor stabilization at the synapse [51]. These findings support the notion that similar behavioural outcomes can be achieved through sex-specific molecular changes, likely reflecting differences in baseline synaptic organization which could be dependent on sex hormone receptors sensitivity. Indeed, myo-inositol has been shown to interact with hormone production and signalling. For example, it has been demonstrated that dopamine and estrogen receptors (ERs) in the nucleus

accumbens trigger similar signalling cascades, ending in the activation of protein kinase A which phosphorylates GluA1 subunits at the serine 845 residue [52]. Furthermore, myo-inositol is a key precursor of phosphoinositides, which regulate multiple aspects of synaptic function, including receptor trafficking, membrane excitability, and intracellular signalling [53]: phosphoinositide-dependent pathways are known to interact with protein kinase A and other kinases involved in AMPA receptor phosphorylation, including GluA1 at serine 845 [54-56]. It is therefore tempting to speculate that, in females, myo-inositol administration can restore ERs pathways converging on AMPA synaptic trafficking, due to sex-dependent different ERs distribution. In this case, future investigations will be needed to establish whether myo-inositol administration can increase the production of estrogen, regularize estrous cycle and modulate synaptic function through ERs- dependent mechanisms in the aged female. Of course, we can't rule out the possibility that inositol is improving other factors, such as insulin sensitivity [57]. Indeed, different reports have shown that inositol administration can ameliorate insulin resistance, a common feature of aging [58], and insulin-resistance has been tightly linked to the development of mood disorders in both laboratory settings and epidemiological studies [59], thus suggesting another potential explanation of our results.

However, several limitations of the present study should be acknowledged. First, while we focused on key synaptic markers implicated in depression and plasticity, additional pre- and postsynaptic proteins, as well as cell-type-specific effects, were not assessed, nor was assessed their functional recovery, as, for instance, increased expression of PSD-95 in bulk lysates may not directly translate to increased synaptic localization and functional insertion. Moreover, although behavioural and molecular analyses suggest a link between synaptic remodelling and mood improvement, causal relationships remain to be established, as our study is mainly descriptive and does not provide a direct mechanism of action. Future studies employing electrophysiological approaches, analysis of spine dynamics and regulation of the trafficking of synaptic proteins will be necessary to fully elucidate the mechanisms underlying the effects of myo-inositol on synaptic plasticity and apathy in aging. Finally, we did not assess hormonal cycle variations in female mice, as estrogen production is heavily reduced with age [60]; however, we cannot exclude a contribution of hormonal fluctuations in our models.

To our knowledge, this is the first study to assess the possibility to employ myo-inositol supplementation for the treatment of LLD. Collectively, our results demonstrate that: chronic myo-inositol administration i) reverts age-related apathetic and altered self-care

phenotype; ii) increases the resilience to additional stress; iii) promotes the expression of synaptic markers in a sex specific manner. By highlighting synaptic remodelling as a convergent substrate for behavioural recovery in aging, these results provide a framework for considering myo-inositol as a candidate intervention for LLD. Furthermore, myo-inositol supplementation is well tolerated in humans, therefore strengthening the translational relevance of our findings. Further work will be required to determine whether myo-inositol may be effective as a stand-alone treatment or as an adjunct to existing pharmacotherapies, and to clarify its translational relevance in different clinical populations.

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Author contribution

RM, NF, FS and GC designed the research, drafted the manuscript and provided critical revisions; DC, AS and LN performed behavioural experiments and analysis; DC, NSI, GN and FN performed molecular experiments and analyses. All authors have reviewed and approved the final manuscript.

Competing Interests

The authors declare no competing interests.

Data availability

Datasets employed in this work are available for download as Supplementary materials.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2026.0255.

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