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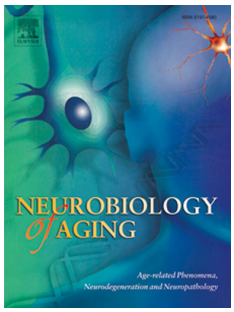

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Andrographolide recovers cognitive impairment in a natural model of Alzheimer's disease (*Octodon degus*)

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Abstract

The social species *Octodon degus* (degu) is the only wild-type South American rodent that develops Alzheimer's-like pathology with age. Here, we evaluated the ability of a natural product (Andrographolide, ANDRO), a diterpene of the labdane family obtained from the Asian plant *Andrographis paniculata*, to recover the cognitive decline in this long-lived animal model. We administered ANDRO to aged degus (56 months old) for 3 months. Additionally, in two control groups (young degus: 12 months old and aged degus: 56 months old) we administrated saline solution as a vehicle. We evaluated cognitive performance through several behavioral tests. We also performed a series of physiological and biochemical analyses (e.g., electrophysiological and immunoblotting assessment) to identify possible mechanisms underlying cognitive performance associated with age. Our results suggest that there is an effect of aging on the loss of cognitive function, and this decrease in cognitive function was also related to a decrease in the synaptic functions and an increase in the main hallmarks of Alzheimer's disease (AD). More importantly, ANDRO treatments showed the following beneficial effects: (1) recovery of spatial memory and learning performance; (2) recovery of synaptic basal transmission; (3) partial or complete protection of certain synaptic proteins; and (4) a specific neuroprotective effect, including the reduction of phosphorylated tau protein and Aβ aggregate maturation in aged degus. Taken together, our results suggest that ANDRO could be used as a potential therapy for AD and support the use of *O. degus* as a natural model in which to study both neural damage associated with aging processes and the behavioral and neuropathological hallmarks of aging-related diseases such as AD.

Key words: *Octodon degus*, Alzheimer's disease, Andrographolide, behavior, cognitive performance

1. Introduction

Alzheimer's disease is characterized by progressive memory loss and neuropathological changes in specific regions of the brain (Takashima, 2009; Duthey, 2013; Selkoe, 2013). The major pathological hallmarks of brains with Alzheimer's disease include the accumulation of neurofibrillary tangles (NFTs) and neuritic plaques, primarily in the hippocampus, cortex, and other brain areas linked to cognitive processes (Glenner and Wong 1984; Götz and Ittner 2008; Takashima, 2009).

The absence of effective treatments that can reverse or stop the progression of Alzheimer's disease motivates the search for new therapeutics (e.g., natural products) (Ng et al., 2015; Serrano et al., 2014). Previous studies have indicated that Andrographolide (ANDRO), a diterpene of the labdane family, is responsible for most of the biological effects of *Andrographis paniculata* (Basak, 1999; Panossian et al., 2000; Iruretagoyena et al., 2005). This molecule has been reported to exert neuroprotective effects against inflammation-mediated neurodegeneration (Wang et al., 2004; Suebsasana et al., 2009), oxidative stress in the brain (Das et al., 2009), and cerebral ischemia (Chan et al., 2010).

Recently, Serrano et al., (2014) showed that ANDRO reduces several neuropathological markers of Alzheimer's disease (including by protecting postsynaptic proteins, reducing Aβ aggregate maturation, and recovering synaptic functions) and recovers spatial memory performance in a transgenic Alzheimer's mouse model of different ages. However, although they are vital tools, the use of these transgenic animal models has been severely criticized because the development of Alzheimer's disease does not progress at the same rate, it does not always affect the same regions of the brain, genetic and/or pharmacological manipulation is needed to reach the intrinsic Alzheimer's pathophysiological state, and the mutated genes are often overexpressed and unable to recapitulate all of the pathological features of this disease (Games et al., 1995; Hock and Lamb 2001; Braidy et al., 2012; Tarragon et al., 2013).

A caviomorph social rodent endemic to Chile, *Octodon degus*, the degu, has gained prominence as the only wild-type South American rodent to develop Alzheimer's-like pathology in older age (Inestrosa et al., 2005; Tarragon et al., 2013; Rivera et al., 2016). Moreover, there is high homology (97.5%) between the human and degu Aβ peptide

sequences (Inestrosa et al., 2005). The aged brains of degus (i.e., age 3-4 years) naturally accumulate senile plaques and neurofibrillary tangles (Inestrosa et al., 2005, 2015), and the affected rodents are insulin resistant, a feature that is common in the clinical manifestations of Alzheimer's patients (Tarragon et al., 2014; Inestrosa et al., 2015). On the other hand, degus exhibit a highly evolved social organization that can recapitulate the richness of human social relationships (Reynolds and Wright 1979; Colonnello et al., 2011a; Rivera et al., 2016). Consequently, the purpose of the present study was to explore the potential effects of ANDRO on memory and synaptic transmission in this novel model animal. We performed an integrative study of the effect of ANDRO in aged degus through the use of behavioral, electrophysiological, and biochemical approaches. We hypothesized that aged degus treated with ANDRO would display improved cognitive abilities compared with aged degus treated with vehicle. To our knowledge, this is the first comparison of the effect of a therapeutic drug to treat cognitive decline in this long-lived animal model.

2. Materials and Methods

2.1. Animals

Adult female degus (56 months old) and young female degus (12 months old) weighing 200 ± 20 g and 153 ± 5 g (mean ± SD), respectively, were obtained from our colony. These animals were all derived from laboratory-bred lines. Degus were randomly divided into three groups (n = 8 per group) and kept in pairs of related and unrelated females housed in clear acrylic aquaria (length x height x depth: 50 x 35 x 23 cm) with a bedding of hardwood chips, and water and food (rabbit commercial pellet; Champion, Santiago, Chile) were provided *ad libitum*. Each cage contained one nestbox made of clear acrylic (22 x 12 x 15 cm). Animals were kept in a ventilated room and exposed to a natural photoperiod and ambient temperature (yearly minimum = 13.4 ± 0.2°C; yearly maximum = 24.9 ± 0.2°C). Under laboratory conditions, degus can live for 8-10 years (Ardiles et al., 2012); however, between 85-95% of degus do not survive to their second year under natural conditions (Ebensperger et al., 2009).

Intraperitoneal (IP) injections of 2.0 mg/kg or 4.0 mg/kg ANDRO in saline vehicle were administered three times per week as described in the literature (Panossian et al., 2000; Hidalgo et al., 2005). Control animals were injected with only vehicle. Twenty-four female degus were used in this study; eight 56 months old degus were used per ANDRO group (2 mg/kg and 4 mg/kg ANDRO), and eight 56 months old degus served as controls. Additionally, eight 12 months old degus (young group) were used as positive control. ANDRO and vehicle were given over three months even while the behavioral tests (see below) were being performed. Each week, we measured body mass and the doses for IP injections were re-calculated. For our study, we did not consider the estrus cycle in the design and performance of the experiments because there is controversy regarding its effects on the learning and memory performance of female rodents (Berry et al. 1997; Stackman et al., 1997; Hornung et al., 2007; Tarragon et al., 2014).

All experiments followed guidelines of the American Society of Mammalogists (Sikes and Gannon 2011) and the National Institutes of Health guidelines (NIH, Baltiomore, MD). All procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile (CBB-121-2013). All efforts were made to minimize animals suffering and to reduce the number of animals used.

2.2. Behavioral testing

Animals underwent four behavioral tests as detailed below. To minimize the effects of behavioral experience on the results, experiments were conducted from less to more intrusive. The order of experiments was as follows: i) open field test, ii) novel object recognition test, and iii) Barnes maze test. Animals underwent one test per day (except the Barnes maze test, which is longer). Since degus are diurnal, all behavioral tests were performed during daytime (between 09:00 and 16:00 h). At the end of each session, the animals were returned to their home cages and the area was wiped clean with a 70% ethanol solution.

158 *2.2.1. Open field test*

159 Animals were observed for 5 min in the open field test. The open field arena consisted of a
160 white Plexiglas box (100 x 100 x 100 cm). The frequency of total crossings and "central
161 crossings" (with a four-paw criterion) were scored (Colonnello et al., 2011b). In addition,
162 the percentage of time in the corners and in the middle arena and the speed and total
163 distance were assessed. At the end of each session, the animals were returned to their home
164 cages and the area was wiped clean with a 70% ethanol solution.

165

166 *2.2.2. Novel object recognition test*

167 This test arena comprised an open box (length x height x depth: 63 x 40 x 30 cm) made of
168 white Plexiglas. For this test, we followed the object recognition protocol used for degus by
169 Tarragon et al. (2014). Briefly, animals were exposed to a 10 min familiarization period
170 and then tested in two consecutive five min assays with a one hour inter-trial interval. For
171 Session 1 (familiarization), two objects ("Object A" and "Object B") were placed in the
172 corners of the home cage and the animal was allowed to freely explore the field for 10 min.
173 Following this period, the objects were removed from the cage and wiped with70% ethanol
174 solution and the test animal was returned to its home cage for one hour. In Session 2 (novel
175 location recognition, NLR), one of the familiar objects (Object B) was moved to an
176 adjacent unoccupied corner. The test animal was then free to interact with the objects for
177 five min. Following this period, the objects were removed from the cage and wiped with
178 70% ethanol solution and the test animal was returned to its home cage for one hour. In
179 Session 3 (novel object recognition, NOR), one of the familiar objects (Object B) was
180 replaced by a different but similar object. We recorded the familiarization and testing times
181 and the time spent exploring each object. "Exploration" time was defined as approaching to
182 within 1 - 3 cm of the object. To quantify NLR and NOR, a recognition index (RI) was
183 calculated as the time spent with Object B divided by the sum of the time spent with Object
184 B and Object A.

185

186 *2.2.3. Barnes maze test*

187 The Barnes maze consisted of a circular 160 cm diameter elevated platform made of white
188 Plexiglas surrounded by a 45 cm high wall. Eighteen circular holes (8 cm in diameter) were

bored through the platform equidistant from each other (16 cm) and 5.5 cm from the outer edge. All holes except the target hole were blocked. A plastic escape box (length x height x depth: 31 x 13 x 16 cm) was positioned under the escape hole. Accurate performance requires subjects to learn and remember the location of the escape hole; therefore, spatial cues (combinations of different colors and shapes—a yellow star, a red square, and a green apple) were placed on the wall of the maze (Kumazawa-Manita et al., 2013). This test has a strong spatial and hippocampus-dependent component (Barnes, 1979). Briefly, the procedure was divided into three phases—habituation, training, and test phases—which were implemented similarly to the methods described by Popović et al. (2010) and Tarragon et al. (2014). Session 1 (habituation) began with placing the animal in the escape box for two min. The animal was then placed near the escape hole and left for one min to escape. If the animal did not enter the escape box, it was gently picked up and helped through the target hole into the escape box, where it was left for two min. Finally, the animal was placed in the center of the maze and left for four min to explore the platform and enter the escape box. If the animal did not enter the escape box, it was placed into the escape box as above and left there for two min. In Session 2 (training), two days after Session 1, we trained each animal for 10 days. In Session 3 (test phase), seven days after Session 2, we exposed the test animals to a memory-retrieval session. Both the training and the test phases consisted of four consecutive four min trials separated by a five min resting phase in the animal's homecage. At the beginning of each trial, the animal was confined for 30 s in a start box in the center of the maze. If the animal did not enter the escape box within the allotted time, it was manually picked up and placed in the escape box, where it remained undisturbed for 2 min. The surfaces of the maze platform were cleaned with 70% ethanol between trials. We recorded the latency to the first visit of the escape hole, the percentage of time in the quadrant of the escape hole. We also analyzed the reference memory errors (every first visit of a non escape hole in each trial) and working memory errors (repeated visits to the same non escape hole in the same trial). To discarded locomotors differences between groups we measured the speed and the distance (in meters) covered from the initiation of exploration of the escape hole to entrance into the escape hole. Search strategies used during reversal trials were categorized into three groups: random, serial and spatial as described by (Inman-Wood et al., 2000; Jašarević et al., 2011).

Briefly, searches were classified as random when localized searches of escape hole were interrupted by center crosses or when no systematic search pattern was discernible. Serial searches were defined as searches of consecutive holes around the maze, and spatial searches were defined as searches following a direct path to the escape hole (see Fig. 3A in the Supplementary Data).

In all cases, a digital video camera (LifeCam Studio Full HD, Microsoft Corp., Redmond, WA) was mounted above the test arena, and the performance of each animal was monitored with image tracking software (HVS Image, Hampton, UK).

2.3. Electrophysiological assessment

The hippocampi of degus were promptly removed and sectioned into 350-μm-thick slices using a vibratome (Leica VT1000S) in ice-cold dissection buffer (5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 212.7 mM sucrose, 10 mM dextrose, 3 mM $MgCl_2$, and 1 mM $CaCl_2$, equilibrated with 95% O_2 and 5% CO_2). The slices were transferred and maintained for 1 h at room temperature in normal artificial cerebrospinal fluid (ACSF), which was similar to the dissection buffer except that sucrose was replaced by 124 mM NaCl, $MgCl_2$was decreased to 1 mM, and $CaCl_2$was increased to 2 mM. All recordings were performed in a submersion recording chamber perfused with ACSF (30 ± 0.5°C; 2 ml/min). Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulating the Schaffer collaterals with 0.2 ms pulses delivered through concentric bipolar stimulating electrodes (FHC) and recorded extra cellularly in CA1 stratum radiatum. Baseline responses were recorded using half-maximum stimulation intensity at 0.033 Hz. Basal synaptic transmission was assayed by determining input-output relationships from fEPSPs generated by gradually increasing the stimulus intensity; a paired-pulse facilitation index was calculated using the equation ((R2-R1)/R1), where R1 and R2 are the peak amplitudes of the first and second fEPSP in an inter-pulse interval of 50 ms. Long-term potentiation (LTP) was induced by theta burst stimulation consisting of four theta epochs delivered at 0.1 Hz. Each epoch in turn consisted of 10 trains of four pulses (at 100 Hz) delivered at 5 Hz.

2.4. Immunoblotting

2.4.1. Western blot analysis

The hippocampi of aged degus (56 months old) treated with ANDRO or control degus (12 months old and 56 months old) treated with vehicle were dissected on ice and immediately frozen at-150°C or processed as previously described (Inestrosa et al., 2013; Serrano et al., 2014). Briefly, the hippocampus tissues were homogenized in RIPA buffer (50 mMTris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitors (50 mMNaF, 1 mM Na_3VO_4 and 30 μM $Na_4P_20_7$) using a Potter homogenizer and were then passed sequentially through different caliber syringes. Protein samples were centrifuged twice at 14,000 rpm at 4°C for 15 min. The protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Twenty and forty micrograms of protein samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated with anti-mouse, anti-goat, or anti-rabbit IgG peroxidase-conjugated antibodies (Pierce, Rockford, IL) and developed using an ECL kit (Western Lightning Plus ECL, PerkinElmer). To analyze the results, all target protein signals were normalized against the loading control (α-Tubulin or β-Actin). In case of the anti-phosphorilated epitopes antibodies the signal was also normalized against the respective total protein signal (E.g: tau-Thr231 and Total tau signals were both normalized against Tubulin and tau-Thr231 signal was also normalized against Total tau signal).

2.4.2. Detection and quantification of Aβ

To determine the concentrations of Aβ peptides, two sandwich enzyme-linked immunosorbent assays (ELISAs) specific for $Aβ_{40}$ and $Aβ_{42}$ were used as previously described (EZBRAIN40, EZBRAIN42; EMD Millipore Corporation, Billerica, MA). Hippocampal homogenates of all animals were diluted to 2μg/μl in homogenization buffer containing protease and phosphatases inhibitors. Approximately 50 μl of diluted homogenate was prepared to measure $Aβ_{40}/Aβ_{42}$ levels according to the manufacturer's instructions. Plates were read at the respective wavelengths on a Metertech 960 ELISA Analyzer.

To detect soluble Aβ oligomers using Western blot analysis, 100μg of protein was separated in a Tris-Tricine buffer system [0.2 M Tris (pH 8.9) as an anode buffer and 0.1 M Tris, 0.1 M Tricine, 0.1% SDS (pH 8.25) as a cathode buffer] and then transferred to a PVDF membrane. The transfers were followed by incubation with the primary antibody anti Aβ-4G8 (Covance) and anti-oligomer-A11 antibody and developed using an ECL kit (Luminata Forte Western HRP Substrate, Millipore Corporation).

2.5. Thioflavin-S (Th-S) staining

To detect amyloid formation, Th-S staining was performed using brain slices mounted on gelatin-coated slides as previously described (Chacon et al., 2004; Toledo and Inestrosa 2010). Slices were dehydrated and rehydrated in xylene and ethanol baths and then incubated in distilled water for 10 min. The slices were then immersed in Th-S solution (0.1% ThS in 70% ethanol) for 5 min, washed twice in 70% ethanol for 30 s, and cover-slipped with mounting medium in the dark. The samples were analyzed using a Zeiss LSM 5 Pascal confocal microscope. The images were analyzed using NIH ImageJ software.

2.6. Immunofluorescence

Immunofluorescence was performed in brain slices as described previously (Cancino et al., 2008; Varela-Nallar et al., 2009). The slices were washed three times in ice-cold PBS and then permeabilized for 30 min with 0.2% Triton X-100 in PBS. After several rinses in ice-cold PBS, the samples were incubated in blocking solution (0.2% bovine serum albumin in PBS) for 1 h at room temperature followed by an overnight incubation at 4°C with primary antibodies. After primary antibody incubation, the slices were extensively washed with PBS and then incubated with Alexa-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) for 2 h at 37°C. The primary antibodies used were rabbit 4G8 and mouse anti-6E10 (Covance, Princeton, NY). The nuclear staining was performed by treating the slices with Hoechst (Sigma-Aldrich, St. Louis, MO). The slices were subsequently mounted on slides using mounting medium and analyzed using a Zeiss LSM 5 Pascal confocal microscope. The images were analyzed using NIH ImageJ software.

2.7. Statistical analysis

All data are presented as the mean ± SE. In the Barnes maze, values are expressed as the mean of the four assays for the test phase (see above). In the NOR test, the recognition index was analyzed. Comparisons among treatments were performed with a one-way ANOVA, and Tukey's post hoc comparison test was used when appropriate at $\alpha = 0.05$. The assumptions of normal distribution and homogeneity of variances were confirmed with a fitting test of the data. We used nonparametric analyses (Mann-Whitney and Kruskal-Wallis) when data could not be transformed to meet these assumptions. Additionally, repeated-measures ANOVA followed by Tukey's post hoc test was used to analyze Barnes maze training data of the different age group and the electrophysiological data. All statistical analyses were performed using the Statistica (StarSoft, Tulsa, OK) software package.

3. Results

3.1. ANDRO recovers the hippocampus-dependent cognitive performance in aged degus

We performed several behavioral task assays to investigate the possible role of ANDRO in aged degus:

3.1.1. Open field test

To evaluate the general state of animals, we performed the open field test. In this context, no significant differences were found between young degus (12 months old) and aged degus (56 months old) treated with the vehicle and aged degus (56 months old) treated with ANDRO (all $P > 0.05$), suggesting a normal general behavior of degus (see Fig. 1 in the Supplementary Data).

3.1.2. Novel object recognition test

We studied the effect of ANDRO on Novel Location Recognition (NLR)/Novel Object Recognition (NOR), which is a double test used to evaluate cognition, particularly

recognition memory. Taking the recognition index (RI) as the dependent variable, the analysis of the effect of ANDRO measured with the NLR trial revealed a significant effect between treatments ($F_{3, 17}$ = 8.471; P < 0.01). Tukey post hoc test indicated a significant effect of age, with a decline in the RI in aged degus compared with young degus (Fig. 1A). The aged control group showed no preference for either the moved or familiar object and thus no significant difference between exploration times for each object ($F_{1, 8}$ = 0.798; P = 0.397; Fig. 1B). More importantly, we observed an increase in the RI in aged degus treated with ANDRO, suggesting that ANDRO treatments improved the spatial working memory in aged degus (Fig. 1A). When we evaluate the exploration times, aged degus treated with ANDRO were able to identify the novel object location significantly better than the aged control ones ($F_{1, 8}$ = 7.615; P = 0.025 and H = 4.364; P = 0.037; Fig. 1B). Similarly, during the NOR assay, we observed a significant difference between treatments ($F_{3, 17}$ = 5.274; P < 0.01). More extensive analyses revealed a significant decrease in the RI in aged degus compared with young animals (Fig. 1C). The aged group showed no preference for the novel object, indicating a lack of memory of the sample object ($F_{1, 8}$ = 0.039; P = 0.847; Fig. 1D). ANDRO treatments significantly increased the RI, moreover, aged degus treated with ANDRO had spend more time with the new object than the familiar ones ($F_{1, 8}$ = 40.52; P < 0.01 and H = 5.28; P = 0.022; Fig. 1D), suggesting a recovery in the memory and predilection for novel experiences in aged degus.

3.1.4. Barnes maze

The Barnes maze test indicated a significant effect of groups ($F_{3, 153}$ = 8.951; P < 0.001) and time ($F_{9, 153}$ = 12.313; P < 0.001), but not of interaction group × time ($F_{27, 1539}$ = 0.428; P = 0.994) on the latency to the first visit of escape hole (Fig. 2A). Tukey test indicated that young animals significantly needed less time than aged control degus to the first visit the escape hole (P < 0.001). Moreover, under ANDRO treatments animals found the escape hole sooner than aged control group (P < 0.001). Latency to the first visit of escape hole decreased over training days in all groups (Fig. 2A), however only animals under ANDRO 4 mg/kg were statistical significant (P = 0.022), during days 3, 7-10. Statistical differences between young and aged animals were found during the nine days of training. Differences between aged degus and ANDRO 2 mg/kg were found during days 3, 4, 6 and 7. More

importantly, during the test phase of the Barnes maze test we found a significant effect of ANDRO on the time to the first visit of the escape hole (H = 8.248; *P* = 0.041). Post hoc analysis revealed that aged degus required approximately five-fold longer time to locate the escape hole compared with young degus, whereas aged animals treated with ANDRO required only twice as long to find the escape hole compared with young animals (Fig. 2B and Fig. 3). When the maze was split into four zones, we found that young and aged degus treated with ANDRO expressed a similar spatial preference for the target area compared with aged degus treated with vehicle (H = 9.108, *P* = 0.028; post hoc analysis; *P* < 0.05, Fig. 2C and Fig. 3).

The analyses of the reference memory errors during training sessions produced similar results. Briefly, a significant effect of groups was present ($F_{3, 153}$ = 19.278; P < 0.001). There was a tendency for a decrease errors as training progressed, ($F_{3, 153}$ = 3.027; P = 0.002), but not in the interaction group × time ($F_{3, 153}$ = 0.0903; P = 1.000; Fig. 2D). Post hoc analysis demonstrated that the aged control group had more reference errors that the young ones (P < 0.001). Interestingly aged degus under both ANDRO treatments committed less errors than the aged control group (P < 0.001). As shown in Figure 2D, we detect statistic differences between young and aged control degus during day 1, 2, and 3. Statistical differences were found between aged control degus and animals treated with ANDRO 4 mg/kg only during day 7. During the test phase of the Barnes maze aged control degus made more errors compared to young and aged degus treated with ANDRO, although such differences did not rise to the level of statistical significance. Working memory errors were analyzed similarly, the analysis showed a significant effect of groups ($F_{3, 153}$ = 37.070; P < 0.001) and time ($F_{3, 153}$ = 2.082; P = 0.034), but no statistical effect in interaction group × time ($F_{3, 153}$ = 0.0903; P = 1.000; Fig. 2E). Tukey test indicated that aged degus committed more errors than young animals (P < 0.001). Moreover, under both ANDRO treatment aged degus committed few errors than aged control degus (P < 0.001). As shown in Figure 2E, post hoc analysis demonstrated that young degus had significant more working memory errors on days 3, 7, and 10. Marginally no significant differences were found during days 5, 8 and 9. During test phase no differences were found between the different groups. Overall, no differences were detected in average speed and total distance traveled during the test phase ($F_{3, 17}$ = 2.874; *P* = 0.07 and $F_{3, 17}$ = 0.423; *P* =

0.739, respectively), indicating that ANDRO treatment did not cause differences in activity levels of aged degus while exploring the maze (see Fig. 2A and 2B in the Supplementary Data).

Random and spatial search strategies showed a significant effect of groups ($F_{3, 153}$ = 11.589; $P < 0.001$ and $F_{3, 153}$ = 3.421; $P = 0.041$) and time ($F_{9, 153}$ = 9.680; $P < 0.001$ and $F_{9, 153}$ = 5.846; $P < 0.001$), but not of interaction group × time ($F_{27, 1539}$ = 0.522; $P = 0.975$ and $F_{9, 153}$ = 0.793; $P = 0.755$), respectively. For serial oriented strategy we observed a significant effect of groups ($F_{3, 153}$ = 5.978; $P < 0.01$), but no significant effect of time ($F_{9, 153}$ = 1.328; $P = 0.277$), or interaction group × time ($F_{27, 1539}$ = 0.693; $P = 0.868$).

The dominant strategy for aged control degus was random across the ten days of training (see Fig. 3B in the Supplementary Data). However, a low proportion of serial and spatial search also were present after day 4 ($P < 0.001$). During test phase aged control degus navigated by using a random strategy (see Fig. 3B in the Supplementary Data). Most young animals started with a random and serial strategy across the two first days of training sessions. After that, they alter their research to a combination of spatial and serial search across the next days ($P < 0.001$), finally after day 8, young degus acquired a more efficient spatial search, to the end of training and during test phase (see Fig. 3C in the Supplementary Data). Whereas, most aged degus treated with ANDRO 2mg/kg alter their search strategy from a combination of the 3 strategies search during the 3 first days to a more spatial oriented strategy by day 6 of training until test phase ($P < 0.001$; Fig. 3D in the Supplementary Data). Finally aged degus under ANDRO 4 mg/kg acquired a combination of random and serial search strategy during first day of training, changing to a combination of serial and spatial oriented strategy during the last days of training sessions ($P < 0.001$) and during test phase (see Fig. 3E in the Supplementary Data).

3.2. ANDRO improves synaptic strength in aged degus but does not have an effect on synaptic plasticity

To assess the effects of aging and the pathological progression of AD hallmarks on synaptic physiology, we performed electrophysiological experiments to measure field excitatory postsynaptic potentials (fEPSPs) in the stratum radiatum of the CA1 area of hippocampal

slices in response to the stimulation of the Schaffer collaterals. As Fig. 4A shows, aged degus exhibited reduced synaptic strength compared with young animals, as measured by the relationship of stimulus strength to fEPSP slope (input-output relationship; repeated-measures ANOVA main effect of age: $F_{3, 128} = 0.903$; $P = 0.462$; Fig. 4B).This difference between young and aged degus increased with increasing stimulus intensities (repeated-measures ANOVA main effect of stimulus: $F_{8, 128} = 38.224$; $P < 0.001$; Fig. 4B). However, this decrease in synaptic strength was recovered after ANDRO treatments, as evidenced by the increase in the fEPSP magnitude in response to increasing stimulus intensities in aged degus (Fig. 4A-B). The paired-pulse facilitation (PPF) index, measured as the ratio between two pulses separated by 50 m, was not affected by either age or the ANDRO treatment (Fig. 4C).

Next, we assessed whether synaptic plasticity was affected at the CA3-CA1 synapses in the aged degus by measuring the long-term potentiation (LTP). Theta burst stimulation (TBS) induces a long-lasting potentiation (60 min) of the fEPSP in young degus; however, the LTP magnitude was significantly decreased after 60 min of induction in aged degus (fEPSP slope increases by TBS: 2.254 ± 0.115r.u. for young degus, n = 4, and 1.732 ± 0.092 r.u. for aged degus, n = 3, Fig. 5). Then, we evaluated whether ANDRO treatment in aged degus would result in improved synaptic plasticity. The ANDRO treatments did not significantly increase LTP magnitude after 60 min of induction by TBS (fEPSP slope increase by TBS: 1.732 ± 0.092 r.u. for aged degus treated with vehicle, n = 3; 1.887 ± 0.186 r.u. for aged degus treated with 2 mg/kg of ANDRO, n = 3; and 1.736 ± 0.077 r.u. for aged degus treated with 4 mg/kg of ANDRO, n = 3, Fig. 5).

3.3. ANDRO recovers the synaptic functions in aged degus

To observe the composition of the synapses, we performed Western blot analysis of the pre- and postsynaptic proteins in the hippocampus of *O. degus*. No consistent differences were observed in presynaptic proteins (Fig. 6A). Furthermore, no differences were observed for synapsin (SYN) in young vs. aged degus treated with vehicle or in aged degus treated with ANDRO (a slight change at 2 mg/kg, Fig. 6B). In the case of the vesicular glutamate transporter 1 (VGluT1) protein, a decrease was observed in aged degus, which

was partially recovered with 2 mg/kg and completely recovered with 4 mg/kg ANDRO (Fig. 6C). Finally, in the case of synaptophysin (SYP), a slight decrease was observed in aged animals; however, ANDRO treatments were not able to recover this effect (Fig. 6D).

In the case of postsynaptic proteins, we observed a clear decrease in aged degus compared with young degus (Fig. 6E). More importantly, the GluN2A subunit of the NMDA receptor was partially recovered with 2 mg/kg and completely recovered with 4 mg/kg ANDRO (Fig. 6F), whereas in the case of postsynaptic density 95 (PSD-95), an opposite effect was observed (Fig. 6G).

3.4. Tau phosphorylation decreases after ANDRO treatments in aged degus

Tau is one of the earliest hallmarks of AD, specifically in its phosphorylated state. Tau has several aminoacid sites that can be targets for post-translational modifications by many kinases, including glycogen synthase kinase 3-β (GSK3β), MAP/microtubule affinity-regulating kinase (MARK), and cyclin-dependent kinase 5 (CDK5). To examine whether ANDRO treatment affects the level of tau phosphorylation in the hippocampus of *O. degus*, Thr231, Ser235and Thr205-Ser202 (AT8) phosphorylation were evaluated. Fig. 7 shows that the phosphorylation of these residues was increased in aged degus (Fig. 7A). Consistent with previous results, ANDRO 2 mg/kg completely decreased these effects for Thr231 and Ser235 (Fig. 7B-C), whereas ANDRO 4 mg/kg significantly reduced all the observed phosphorylated tau epitopes (Fig. 7D). Also, a shift in the molecular weight of total tau is observed, this may be due to multiple phosphorylations presented at this protein which causes a slight increase in its molecular weight.

3.5. Aβ40 and Aβ42 peptides decrease after ANDRO treatments in aged degus

To determine whether ANDRO treatments could interfere in the processing of the amyloid precursor protein (APP), we analyzed the soluble $A\beta_{40}$ and $A\beta_{42}$ peptides in the hippocampus of *O. degus* using an ELISA. Fig. 8 shows that aged degus presented an increased level of $A\beta_{42}$ peptide and that ANDRO treatments decreased this effect, especially at the 4 mg/kg concentration.

To observe the Aβ soluble oligomers and other Aβ species levels in the hippocampus of degus, we performed a Western blot analysis using the 4G8 antibody. Fig. 9 shows that higher levels of low-molecular-weight (36 and 42 kDa) Aβ oligomers are present in the hippocampus of young and aged degus treated with vehicle (Fig. 9A); specifically, we found an increase of 34-kDa Aβ oligomers in aged degus (Fig. 9B). ANDRO treatments significantly decreased the levels of all low-molecular-weight Aβ oligomers (Fig. 9B-D). Together, these results indicated that ANDRO treatment decreased the levels of$A\beta_{42}$ peptide and the Aβ oligomers.

3.6. ANDRO reduces Aβ aggregates in the brain of aged degus

To determine whether treatment with ANDRO could affect the Aβ burden, we performed thioflavin (Th-S) staining in the hippocampus of young and aged degus (Fig. 10A). In young animals, we did not observe the formation of insoluble forms of Aβ (senile plaques). However, we observed several plaques in the aged degus. ANDRO treatments significantly decreased the number of senile plaques in the hippocampus in a concentration-dependent manner (Fig. 10B). We also studied the expression of Aβ aggregates (soluble and insoluble forms of Aβ) using the 6E10 antibody, which is reactive to a specific amino acid sequence, 1-16 of the Aβ peptide (Zhang et al., 2012). In young degus, we did not observe any 6E10, but high levels of expression were found in the aged degus. ANDRO treatments significantly decreased the 6E10 levels in aged degus (Fig. 10C-D). We also used a second antibody, 4G8, which is specific for another amino acid sequence, 17-24 of the Aβ peptide (Thakker et al., 2009). Similarly to 6E10, young degus did not show expression of 4G8 compared with aged degus, and we found a significant decrease in the Aβ aggregates in the hippocampus of aged degus treated with ANDRO (Fig. 10E-F).

4. Discussion

Aging is a progressive functional decline characterized by a gradual deterioration of physiological function, including changes in anatomy, endocrine systems, neural circuitry, and behavior (Shoji and Mizoguchi2010; Lopez-Otin et al., 2013). There is evidence for a

causal role of the aging process in the development of neural and psychopathologies such as Alzheimer's disease (AD) (Galluzzi et al., 2008; Kadish et al., 2009; Duthey, 2013; Scheff et al., 2014). AD is the most common form of dementia, and it is characterized by progressive memory loss and neuropathological changes in specific regions of the brain that lead to death (Selkoe, 2013). Although no effective cure exists for AD, recent clinical studies have proposed new natural products to treat and prevent the progression of this neurodegenerative disease (Ng et al., 2015). Among these products, Andrographolide (ANDRO) seems to be a good candidate. In recent years, several studies using transgenic mouse AD models have examined the positive role of ANDRO (Serrano et al., 2014; Tapia-Rojas et al., 2015; Varela-Nallar et al., 2015). However, these transgenic animal models rely on genetic manipulations and are unable to recapitulate all of the pathological features of AD (Hock and Lamb 2001; Inestrosa et al., 2005; Braidy et al., 2012).

Recently, *Octodon degus* has been identified as a very valuable model for research in neurodegenerative disease associated with aging (Braidly et al., 2012; Tarragon et al., 2013; Rivera et al., 2016). Degus spontaneously develop neuropathological hallmarks of AD after 3-4 years of age (Inestrosa et al., 2005). Moreover, degus between 12 and 36 months naturally develop the neuropathological hallmarks of AD (i.e., accumulation of Aβ oligomers and phosphorylated tau proteins) and display impairment in spatial and object recognition memory and decreased synaptic function (Ardiles et al., 2012). In the present study, we evaluated ANDRO using this natural model of sporadic AD.

Overall, our results suggest that there is an effect of aging on the loss of cognitive functions; aged degus (56 months old) treated with vehicle showed decreased cognitive function compared with young degus (12 months old) treated with vehicle. This decrease in cognitive function was also associated with a decrease in the synaptic functions and an increase in the main hallmarks of AD. More importantly, ANDRO treatment had the following effects: (1) recovery of spatial memory and learning performance; (2) protection of postsynaptic proteins and recovery of synaptic strength; and (3) a specific neuroprotective effect, including the reduction of phosphorylated tau protein and Aβ aggregate maturation in aged degus (56 months old).

A major clinical manifestation of age-related disease is the decline in cognitive capacities; we first evaluated whether ANDRO treatment affected this response in aged

561 degus (56 months old). To study the general behavior of degus, we performed the open
562 field test. We performed a novel object recognition test to evaluate cognition, particularly
563 recognition memory, and finally, we used the Barnes maze test to study spatial learning and
564 memory, processes that both depend, in part, on hippocampal structure (Sunyer et al., 2007;
565 Kumazawa-Manita et al., 2013; Rosenfeld and Ferguson 2014).

566 During the open field test, neither group showed differences compared with young
567 control (12 months old) degus, suggesting normal general behavior (Fig. 1 in the
568 Supplementary Data). In the novel object/local recognition test, we observed a decrease in
569 spatial working memory in aged control (56 months old) degus compared with young
570 control (12 months old) degus. More importantly, we observed an increase in spatial
571 working memory in degus treated with ANDRO. For both NLR and NOR sessions (Fig. 1A
572 and 1C), we observed a significant increase in the recognition index (RI) in degus treated
573 with ANDRO, a result that confirms a recovery of recognition memory (Antunes and Biala
574 2012). During NLR sessions aged degus (control and ANDRO groups) spend more time
575 exploring the objects than young ones (Fig. 1B), moreover during NOR session no
576 differences in the exploration time between groups was found (Fig. 1D), suggesting that
577 aged degus present similar motivation to explore the objects compared to the young ones,
578 so the differences that we observed are not being interfered by this factor. With regard to
579 the NOR test, Ardiles et al. (2012) demonstrated that aged degus cannot recognize the
580 novel object, unlike animals of different ages. The Barnes maze test is a highly
581 hippocampal-dependent spatial learning task used to assess reference memory in rodents
582 (Kennard and Woodfruff-Pak 2011). Animals with cognitive impairments associated either
583 to normal aging or neurodegenerative pathology, exhibit impairment performance,
584 indicated by increased latency and error rate to find the escape hole, compared to control
585 animals (Huang and Kandel 1995; Barreto et al., 2010). In this way, the latency to the first
586 visit of the escape hole, one of the most widely used measure of learning in the Barnes
587 maze (Harrison et al., 2006; Patil et al., 2009), was the most sensitive for detecting
588 differences between aged control degus (56 months old) and aged degus treated with
589 ANDRO. The training sessions of Barnes maze show both, young (12 months old) and
590 aged control degus, progressively reduce the time to the first visit to the escape hole
591 through the consecutive days (Fig. 2A). Moreover, aged degus under ANDRO treatments

reduce significantly the latency to the first visit compared with aged control degus (Fig. 2A). Results on the test phase (i.e., long term retention, measured 7 days after the last training trial) show a significant increase in latency time to find the escape hole for aged degus treated with vehicle compared with the young group, suggesting impaired long-term memory retention in the aged animals. In contrast, aged degus treated with ANDRO presented improved latency time. ANDRO groups were able to find the escape hole in a similar time as young degus treated with vehicle (Fig. 2B and Fig. 3). Additionally, these results were confirmed by the percentage of time spent in the quadrant of the escape hole (Fig. 2C), suggesting that ANDRO recovers spatial learning and memory.

Analysis of reference memory and working memory errors across 10 days of training revealed a significant effect, indicating a decrease in the number of errors for all groups (Fig. 2D and Fig. 2E), and that all groups learned to use the spatial cues to find the escape hole. Additionally, there was a significant effect of ANDRO treatments, these animals committed significantly less number of errors than aged control degus (Fig. 2D and Fig. 2E). No significant interaction was presented for both error analyses. During retention phase, aged animals injected with vehicle committed more errors that young degus and aged degus under ANDRO treatments, although such differences did not rise to the level of statistical significance, indicating that ANDRO treatments had no effect on long term retention in the Barnes maze. This last result may be caused by the fact that aged control degus, if well made more errors, also exhibited greater intra-individual differences. Additionally, measurement of number of errors were not sensitive enough to detect learning impairment on the test phase of Barnes maze, because degus, similar to other rodents, may be more likely to explore other holes instead of entering the escape hole, even when the location of escape hole has been learned (Grootendorst et al., 2001).

The Barnes maze revealed differences in the search strategies used by female degus. In general the strategy was dependent of groups and the day of training session. In this way, young female degus (12 months old), tend to change from a combination of the 3 search strategies, used at the beginning of training, to a more frequently spatial oriented strategy during the test phase, in accordance with Popović et al. (2010). Aged degus treated with vehicle (56 months old) failed to shift to the spatial-oriented strategy by the end of training or during test phase, suggesting that aged animals present alterations in cognitive

or attentional abilities. Moreover, aged degus under ANDRO treatments navigated by using a combination of the 3 strategies during first day of training, followed by a prevalence of serial search during the last day of training and also during test phase for aged degus treated with ANDRO 2 mg/kg and a prevalence of combination of serial and spatial strategy for degus under treatment of ANDRO 4 mg/kg.

Its known that animals tend to alter their navigation strategy from random search, used at the beginning of training, to a more efficient spatial orientated search when training progresses (Harrison et al., 2006; Jašarević et al., 2011). The spatial strategy is cognitively more demanding because requires the use of multiple relationships among extra-maze cues to guide the animal to the escape hole (Bach et al., 1995; Inman-Wood et al., 2000). Whereas, serial strategy is less efficient because requires an animal to remember to search each consecutive hole (Inman-Wood et al., 2000), however it can support considerably better escape performance and also have less error than animals that randomly search for the escape hole (Gallagher et al., 1993; Harrison et al., 2006). Taken together; these results suggest that aged degus (56 months old) under both ANDRO treatments showed significant improvements in reaching the escape hole quickly and efficiently, and more importantly ANDRO treatment, particularly ANDRO 2 mg/kg, was able to compensate the alteration in cognitive or attentional abilities observed in aged control degus.

Together, these data support the notion that aged animals perform poorer performance in memory task in comparison with young animals, similar to the reported by Ming and Song (2005). More important, aged degus undergoing ANDRO treatment showed restored cognitive function approximating that of young degus (12 months old).

For our design, we did not take into consideration the effect of the females' hormonal fluctuation during the behavioral test because there is controversy regarding the effects of this cycle on the memory of female rodents. In a recent study with young degus (16months), the authors reported sex differences during the training phase in the BM when they considered the diestrus phase of females (i.e., period in which females do not differ significantly from males). However, in the same study, during the test phase, there were no significant gender differences in memory capacity (Popović et al., 2010). Similarly, Frye (1995) did not found hormone-dependent differences during the training phase in the water maze task in rats. Other studies performed throughout the estrus cycle did not report

differences during the acquisition phase or in the performance of the working memory task by female rats in the water maze and the radial maze (Berry et al., 1997; Stackman et al., 1997). Our results indicate that no differences intra-groups were detected in the performance of behavioral tests.

Because behavioral analyses alone may not be able to determine the mechanisms that underlie the observed cognitive impairment associated with age and the subsequent recovery observed with ANDRO treatment, we performed a series of functional and biochemical analyses. A direct measure of age-dependent changes in neuronal activity and plasticity is provided by electrophysiological studies in hippocampal slice preparations. In electrophysiological experiments, we observed a decrease in the basal synaptic transmission, as measured by the I-O relationship, in aged control (56 months old) degus compared with young control (12 months old) degus, which is in agreement with previous findings in aged rats (Norris et al., 1998; Kumar and Foster 2013), mice (Weber et al., 2015), and degus (Ardiles et al., 2012). As a correlate test of impaired learning and memory in aged degus, we measured hippocampal LTP and observed a significant reduction in its magnitude in the aged control degus compared to the young degus. Previous studies of the effects of aging on TBS-induced hippocampal LTP have produced different results; some reports showed age-dependent deficits, whereas others did not (Deupree et al., 1993; Barnes et al., 1996; Norris et al., 1996; Rosenzweig et al., 1997; Bach et al., 1999; Rosenzweig and Barnes 2003). However, in degus, aging is detrimental to the magnitude of hippocampal LTP, which was explained by the concomitant increase in the pathological hallmarks of AD, including oligomeric forms of Aβ peptide (Ardiles et al., 2012). Interestingly, ANDRO treatments improved the synaptic basal transmission (Fig. 5), which is consistent with the recovery in cognition, but the treatments did not have significant effects on LTP reduction. This finding is consistent with previous studies showing that ANDRO treatment does not affect the induction of LTP in young or aged wild-type mice (Serrano et al., 2014). LTP involve several of the molecular and structural changes that are underling to the processes of learning and memory, however in our hands, the induction and magnitude of LTP is not affected by ANDRO treatment. Most studies, report a positive correlation between LTP and spatial memory, however, the dissociation between LTP and distinct forms of spatial memory has been observed in several different mouse strains

(Zamanillo et al., 1999; Kaksonen et al., 2002; Pineda et al., 2004; Rutten et al., 2008; Meiri et al 1998). Interestingly, the group of Kim et al., recently showed that the oral administration of 4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid (EPPS) substantially reduces hippocampus-dependent behavioral deficits but not alters the LTP induction in APP/PS1 transgenic mouse model (Kim et al., 2015). Another study shown that the use of a volatile anesthesic, sevoflurane, improves cognitive performance in mice, but does not influence LTP induction and magnitude in the hippocampus (Haseneder et al., 2013).

The decrease in the synaptic strength, plasticity, and cognition could be explained by the concomitant decrease in several synaptic proteins, such as PSD-95 (Zhang and Lisman 2012), GluN2A (Wang et al., 2004), vGlutT1 (Balschun et al., 2010), and SYP, which are important in excitatory synaptic transmission. All these proteins are essential for excitatory synaptic transmission; PSD-95 affects synaptic maturation, specifically the amplitude of the excitatory postsynaptic currents (EPSCs), a direct measure that indicates the synaptic strength, but it is not required for functional changes during an early LTP (Ehrlich et al., 2007; Zhang and Lisman 2012; Vallejo et al., 2016). Another primary factor in the synapse is the NMDA receptor, which is strongly involved in synaptic strengthening and weakening in response to activity patterns; therefore, the 2A subunit of the NMDA receptor has been strongly associated with the LTD induction process (Shipton and Paulsen 2016).

Despite these synaptic changes, the SYN level did not show significant changes in the aged degus, suggesting that this protein is not affected by aging and/or the progression of AD hallmarks in this animal model. Thus, the restorative properties of ANDRO may be related to its ability to increase the levels of these synaptic proteins to levels similar to those in the young control degus. Interestingly, ANDRO was not able to restore SYP levels in the aged degus. SYP is among the most abundant and conserved synaptic vesicle proteins, but its function is not fully understood (Südhof, 1995). Several studies suggest that SYP function is largely redundant because its knockout (KO) model does not affect neurotransmitter release and plasticity (McMahon et al., 1996). However, other studies suggest that SYP has a role in the induction of LTP (Mullany and Lynch 1998; Li et al., 2012), which may be more pronounced if other presynaptic components are also affected

(Janz et al., 1999). The fact that ANDRO could not restore SYP levels in the aged degus (and most likely other synaptic proteins not evaluated in this study) may indicate a pathological mechanism that is unaffected by ANDRO and thus maintains the induction of LTP in treated aged degus. Consistent with these findings, Serrano et al., (2014) showed that in a double transgenic model, 2mg/kg ANDRO was not able to restore the level of presynaptic proteins, including SYP.

In agreement with previous observations (Ardiles et al., 2012), the hippocampus of aged degus (56 months old) showed an increase in several AD hallmarks, including all the phosphorylated tau epitopes evaluated (Thr231, Ser235, Thr205, and Ser202) and the levels of both the $A\beta_{40}$ and $A\beta_{42}$ peptides. Interestingly, the $A\beta_{42}$ peptide is the most toxic in the brain (Giese, 2012; Bodani et al., 2015). Additionally, we assessed the levels of Aβ soluble oligomers; the low-molecular-weight (approximately 36kDa) oligomers were also increased in aged degus compared to young degus (12 months old). Experimental data using transgenic animal models demonstrate that low-molecular-weight Aβ oligomers can affect neuronal synapses (e.g., attenuation of LTP, induction of LTD) (Walsh and Selkoe 2007; Hayden et al., 2013). Moreover, Cleary et al., (2005) showed that soluble oligomeric forms, including trimers and dimers, were sufficient to produce impaired cognitive functions without inducing permanent neurological deficits. Furthermore, in a deeper analysis, we observed that both Aβ total aggregates (soluble and insoluble) and the Aβ insoluble forms, commonly known as senile plaques, are increased in the aged degus compared with the young degus, in concordance with our results for Aβ peptides and soluble oligomers. An important finding of our work is that the recovery in cognitive performance observed in ANDRO treatments was also associated with a significant reduction of tau protein phosphorylation, Aβ peptides, soluble Aβ oligomers, Aβ aggregates, and Aβ plaques, which is in agreement with previous studies in AD transgenic animal models. Another interesting observation is that in the study of Serrano et al., (2014), ANDRO treatment reduced the levels of Aβ aggregates only in the young group (7 months old) and not in the mature group (12 months old), suggesting that ANDRO prevents Aβ aggregation in the early stages of AD development. These results are consistent with the idea that 56 months old degus correspond to an early stage in the progression of sporadic AD because we were able to reduce Aβ levels with ANDRO treatments.

Part of the molecular mechanism mediating the effects of ANDRO observed in our work may involve the modulation of other previously described signaling pathways (Godoy et al., 2014). For instance, ANDRO inhibits certain pathways related to inflammation and apoptosis, including Akt, NF-κB, and MAPK signaling (Hidalgo et al., 2005; Carretta et al., 2009; Lu et al., 2011). More recently, we showed that ANDRO could inhibit GSK-3β activity through two mechanisms: via non-ATP competitive inhibition and by favoring a misbalance in its autoregulation (Tapia-Rojas et al., 2015), leading to downstream activation of the canonical Wnt pathway, which has a key role in AD pathogenesis (Inestrosa and Arenas 2010). Additional experiments using the degu as a study model should be performed to determine the potential effects of ANDRO on Wnt signaling modulation.

In summary, our results support the potential use of ANDRO to treat AD. Using the degu, a social long-lived animal, enabled us to understand the processes underlying the cognitive decline associated with brain aging and neurodegenerative disorders and to observe the subsequent recovery with ANDRO treatment. Our results validate *O. degus* as a natural model in which to study both neural damage associated with aging processes and the neuropathological hallmarks of aging-related diseases such as AD.

Disclosure statement

The authors have no conflicts of interest to disclose.

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Figure Legends

1. NLOR test in young control (12 months old: 12-mo-old; n = 6) and aged control (56 months old: 56-mo-old; n = 5) degus treated with vehicle or aged degus (56-mo-old) treated with 2 mg/kg and 4 mg/kg of ANDRO (n = 5 respectively). (A) Analysis of the recognition index for the "local recognition" trial (time spent exploring the novel location object/time spent exploring the novel location and the familiar location objects). (B) Average exploration time for novel vs. familiar objects location. (C) Analysis of the recognition index for the "object recognition" trial (time spent exploring the novel object/time spent exploring the novel and the familiar objects). (D) Average exploration time for novel vs. familiar objects. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *P < 0.05, **P < 0.01, with Tukey's post hoc comparison.

2. Barnes maze in young control (12 months old: 12-mo-old; n = 6) and aged control (56 months old: 56-mo-old; n = 5) degus treated with vehicle or aged degus (56-mo-old) treated with 2 mg/kg and 4 mg/kg of ANDRO (n = 5 respectively). (A) Learning curve of latency of the first visit to escape hole through the 10 days training sessions and test phase. (B) Latency to first visit of the escape hole across test phase. (C) Percentage of time spent in the quadrant with the escape hole across test phase. (D) Learning curve of the reference memory errors through the 10 days training sessions and test phase. (E) Learning curve of working memory errors through the 10 days training sessions and test phase. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *P < 0.05, **P < 0.01, with Tukey's post hoc comparison.

3. Paths taken by representative animals (e.g., close to the group mean) of the latency to the first visit of the escape hole. (A) Young control (12 months old: 12-mo-old; n = 6) degus treated with vehicle, (B) aged control (56 months old: 56-mo-old; n = 5) degus treated with vehicle, (C) aged degus (56-mo-old; n = 5) treated with 2 mg/kg ANDRO, (D) aged degus (56-mo-old; n = 5) treated with 4 mg/kg ANDRO. The gray area represents the quadrant of the escape hole. The escape hole is indicated in black.

4. Impaired hippocampal synaptic plasticity in *O. degus*. (A) Representative traces of fEPSP at different stimulus intensities from young control (12 months old: 12-mo-old) and aged control (56 months old: 56-mo-old) degus treated with vehicle or aged degus (56-mo-old) treated with 2 mg/kg and 4 mg/kg ANDRO (scale bars: 0.1 mV, 10 ms). (B) Input-output curves for different groups of degus. (C) Plot of paired-pulse facilitation (PPF) between groups. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *P < 0.05, **P < 0.01, with Tukey's post hoc comparison. 2-3 hippocampal slices was used per animals. 3 animals were used per group.

5. Impaired hippocampal synaptic plasticity in *O. degus*. Representative fEPSPs recorded 1 min before TBS (1) and 60 min after TBS (2). LTP protocol was delivered at the time indicated by the arrow. Averaged LTP magnitudes during the last 10 min of recording in different groups of degus. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *P < 0.05, **P < 0.01, with Tukey's post hoc comparison. 2-3 hippocampal slices was used per animals. 3 animals were used per group. r.u. refers to relative units.

6. Treatment with ANDRO modified synaptic protein levels in the hippocampus of *O. degus*. (A) to (D) represent presynaptic protein. (A) Representative Western blot analysis. Relative levels of (B) synapsin (SYN), (C) Vesicular Glutamate Transporter 1 (VGluT1), and (D) synaptophysin (SYP). (E) to (G) represent postsynaptic protein. (E) Representative blot analysis. Relative levels of (F) GluN2A and (G) PSD-95 from young control (12 months old: 12-mo-old) and aged control (56 months old: 56-mo-old) degus treated with vehicle or aged degus (56-mo-old) treated with 2 mg/kg and 4 mg/kg ANDRO. Each lane represents samples from a different animal. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *P < 0.05, **P < 0.01, with Tukey's post hoc comparison ($n \geq 3$).

7. Treatments with ANDRO reduced tauphosphorylation levels in the hippocampus of *O. degus*. (A) Representative Western blot analysis of tau phosphorylation (age and treatment with vehicle or ANDRO above lanes). Relative levels of (B) Threonine 231, (C) Serine

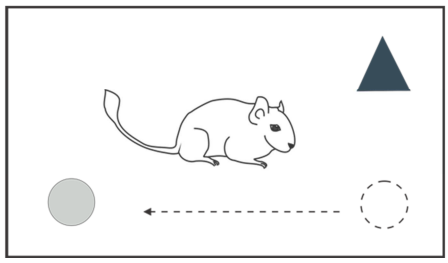
235, and (D) both Serine 202 and Threonine 205 (AT8) in hippocampal lysates from young control (12 months old: 12-mo-old) and aged control (56 months old: 56-mo-old) degus treated with vehicle or aged degus (56-mo-old) treated with 2 mg/kg and 4 mg/kg ANDRO. Each lane represents samples from a different animal. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *P < 0.05, **P < 0.01, with Tukey's post hoc comparison (n ≥ 3).

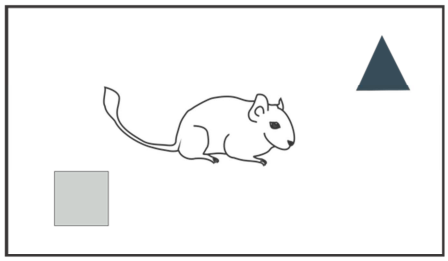
8. Treatment with ANDRO reduced soluble $A\beta_{40}$ and $A\beta_{42}$ peptide levels in the hippocampus of *O. degus*. Real values from ELISAs of (A) $A\beta_{40}$ and (B) $A\beta_{42}$ peptide performed on hippocampal lysates from young control (12 months old: 12-mo-old) and aged control (56 months old: 56-mo-old) degus treated with vehicle or aged degus (56-mo-old) treated with 2 mg/kg and 4 mg/kg ANDRO. ELISA assay performed using 50ul of total soluble protein fraction for the detection of soluble forms of $A\beta_{40}$ and $A\beta_{42}$. Each lane represents samples from a different animal. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *P < 0.05, **P < 0.01, with Tukey's post hoc comparison (n ≥ 3).

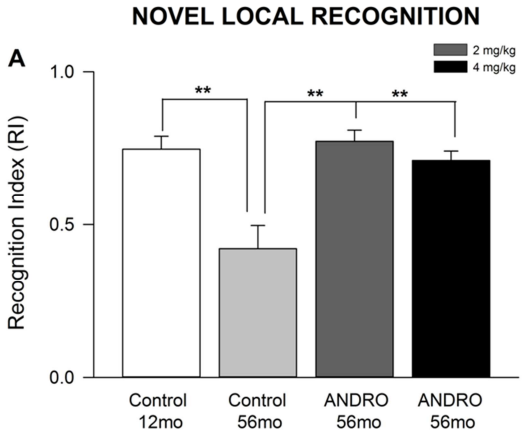
9. Aged degus treated with ANDRO present lower levels of low-molecular-weight Aβ species. (A) Representative image of the Western blot analysis. Protein samples (100 µg) of each animal were separated in a Tris-Tricine gel, transferred onto a PVDF membrane, and incubated with the anti Aβ-4G8 antibody. Densitometry analysis of (A) three molecular weights from young control 12-months-old degus (white bars), aged control 56-months-old degus (light gray bars), aged degus treated with ANDRO 2 mg/kg (dark gray bars), and aged degus treated with ANDRO 4 mg/kg (black bars). (B) 34kDa, (C) 43kDa, (D) 55kDa. Each lane represents samples from a different animal. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *P < 0.05, **P < 0.01, with Tukey's post hoc comparison (n ≥ 3).

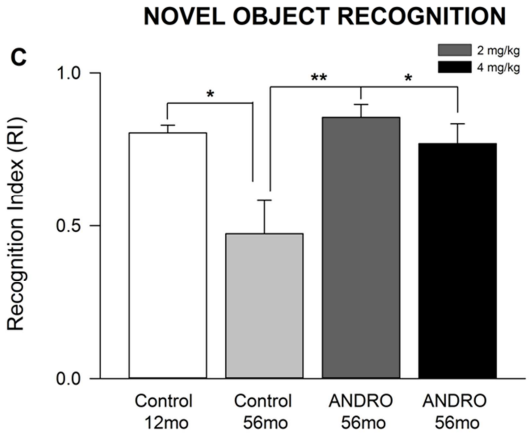
10. Representative immunofluorescence for Aβ in the hippocampus of young degus (12 months old: 12-mo-old) and aged degus (56 months old: 56-mo-old) treated with vehicle and aged degus (56-mo-old) treated with ANDRO. (A) Detection of Aβ insoluble forms

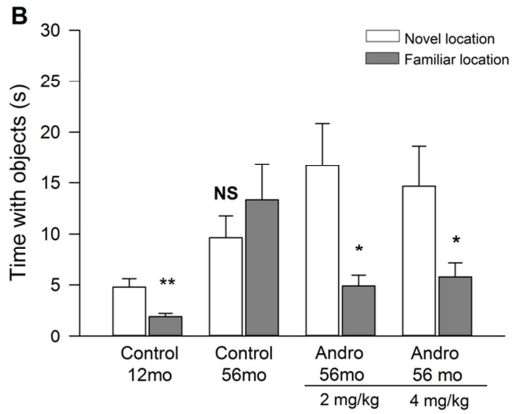
(Aβ fibrillary species/ Aβ plaques) using thioflavin-S stain. (B) Quantification of (A) by area (μm^2). (C) Detection of Aβ aggregates (soluble and insoluble species of Aβ) by immunostaining using anti-Aβ 6E10 antibody. (D) Quantification of (C) by area (μm^2). (E) Detection of Aβ aggregates (soluble and insoluble species of Aβ) by immunostaining using anti-Aβ 4G8 antibody. (F) Quantification of (E) by area (μm^2). The dotted squares indicate the site of magnification. Each lane represents samples from a different animal. Results are expressed as mean ± S.E. Asterisks indicate significant observed differences: *$P < 0.05$, **$P < 0.01$, with Tukey's post hoc comparison. Scale bar: 100 and 40 μm, $n \geq 3$.

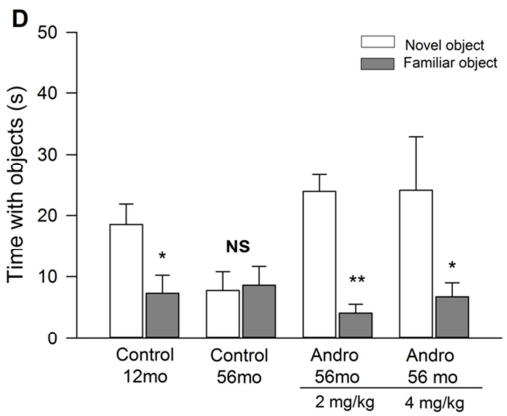


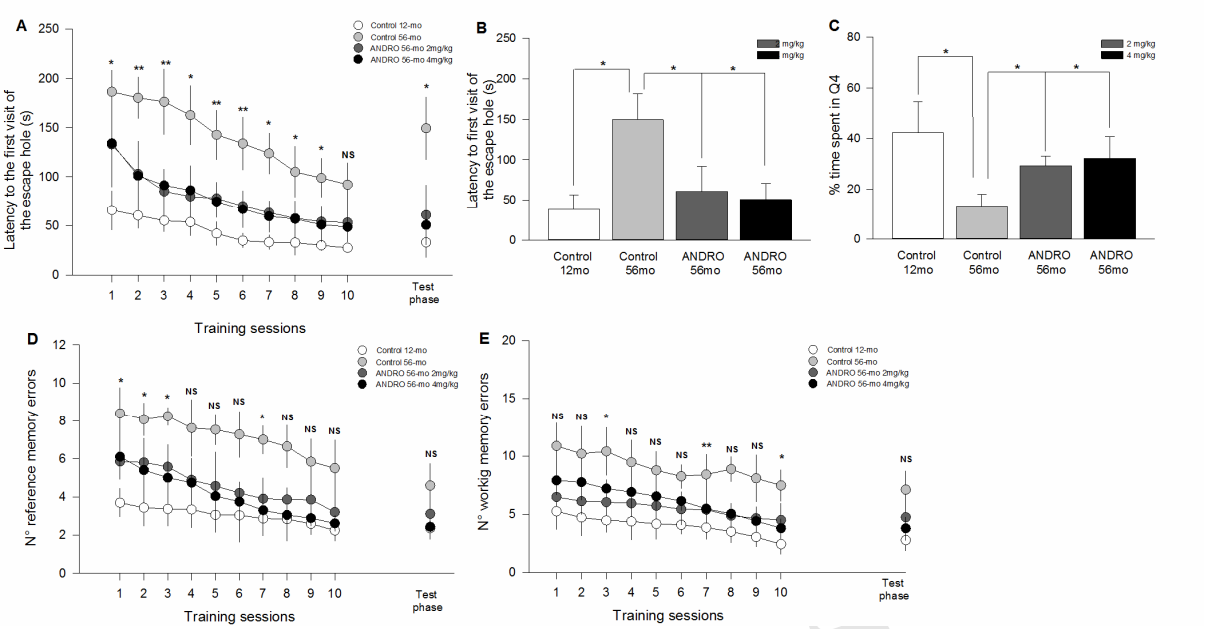


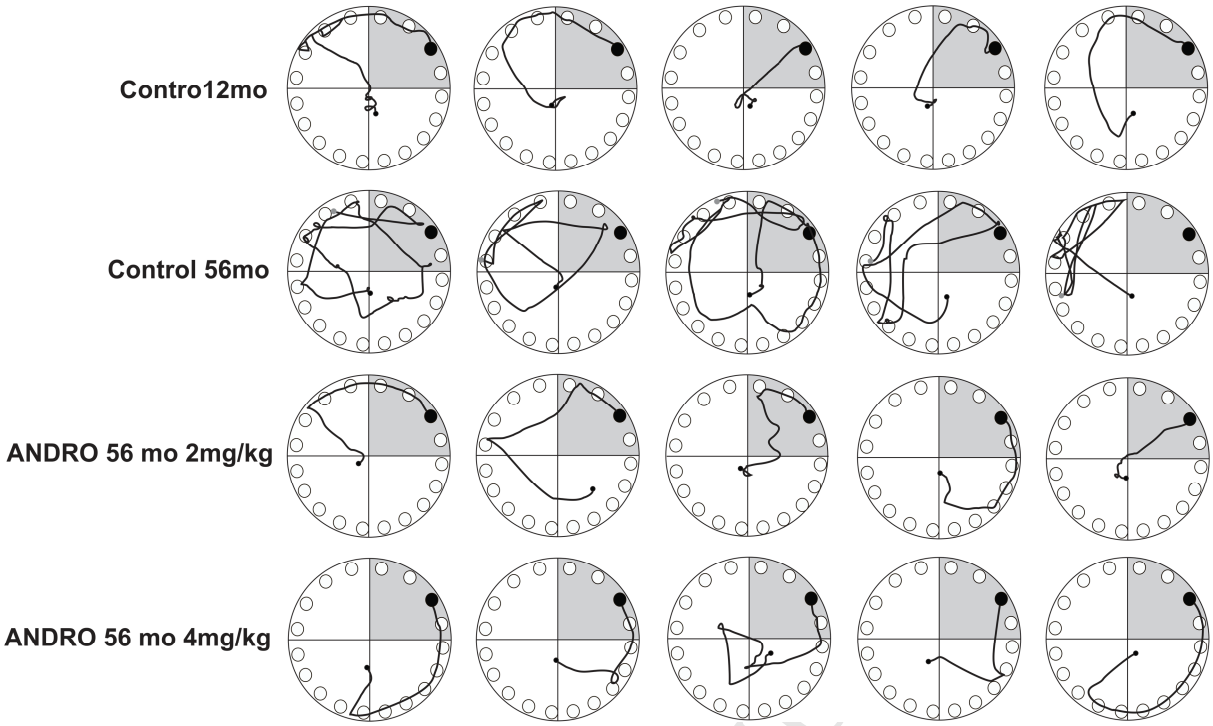


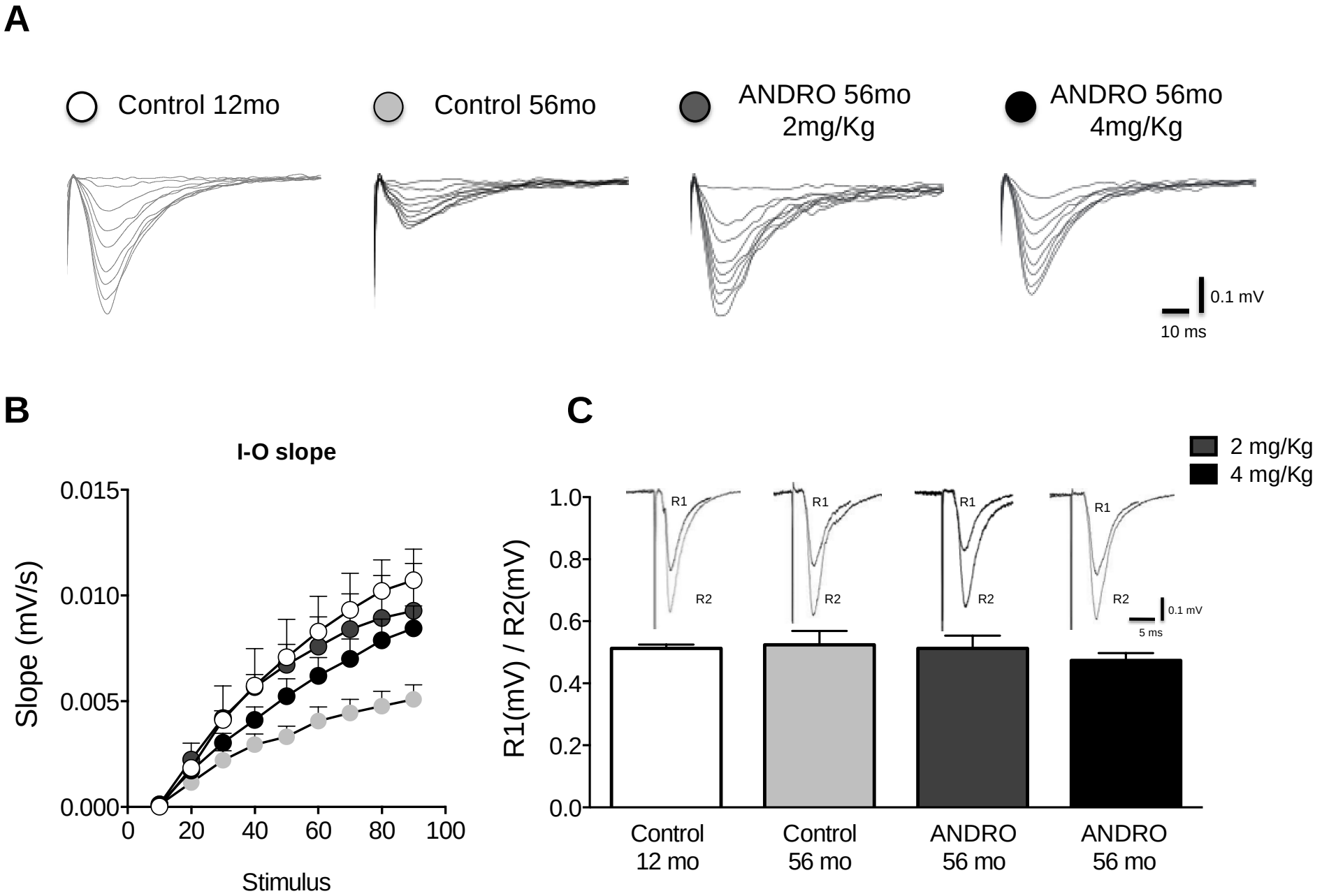


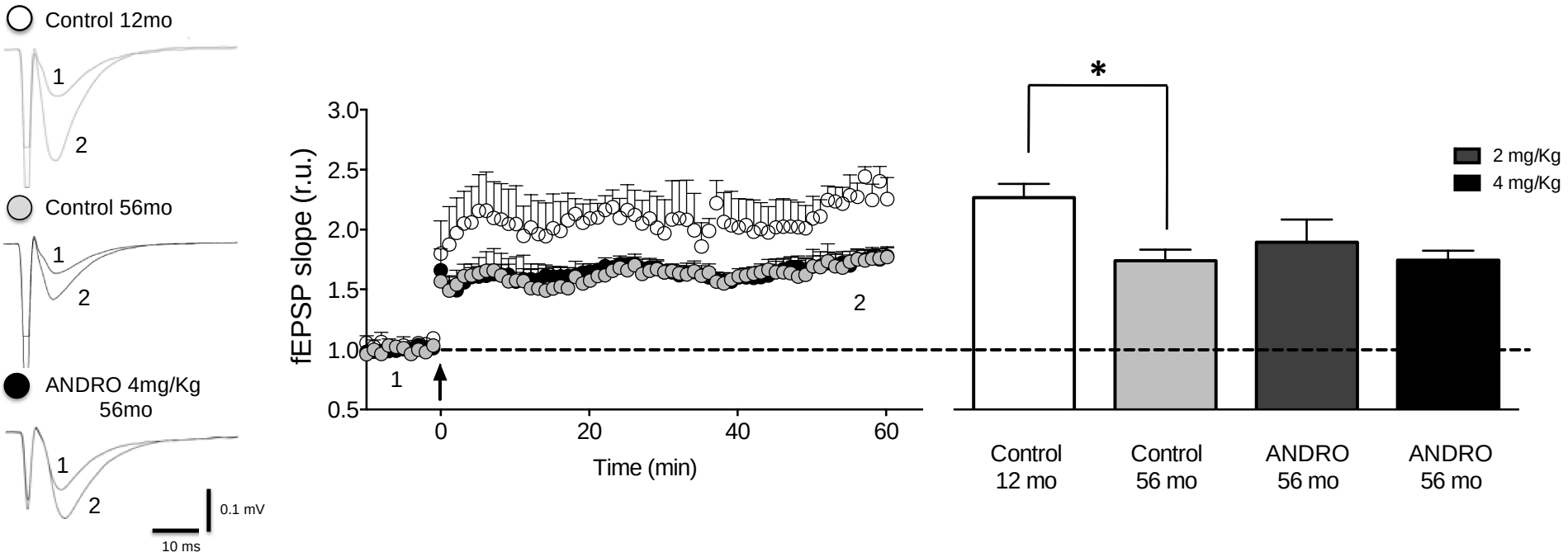


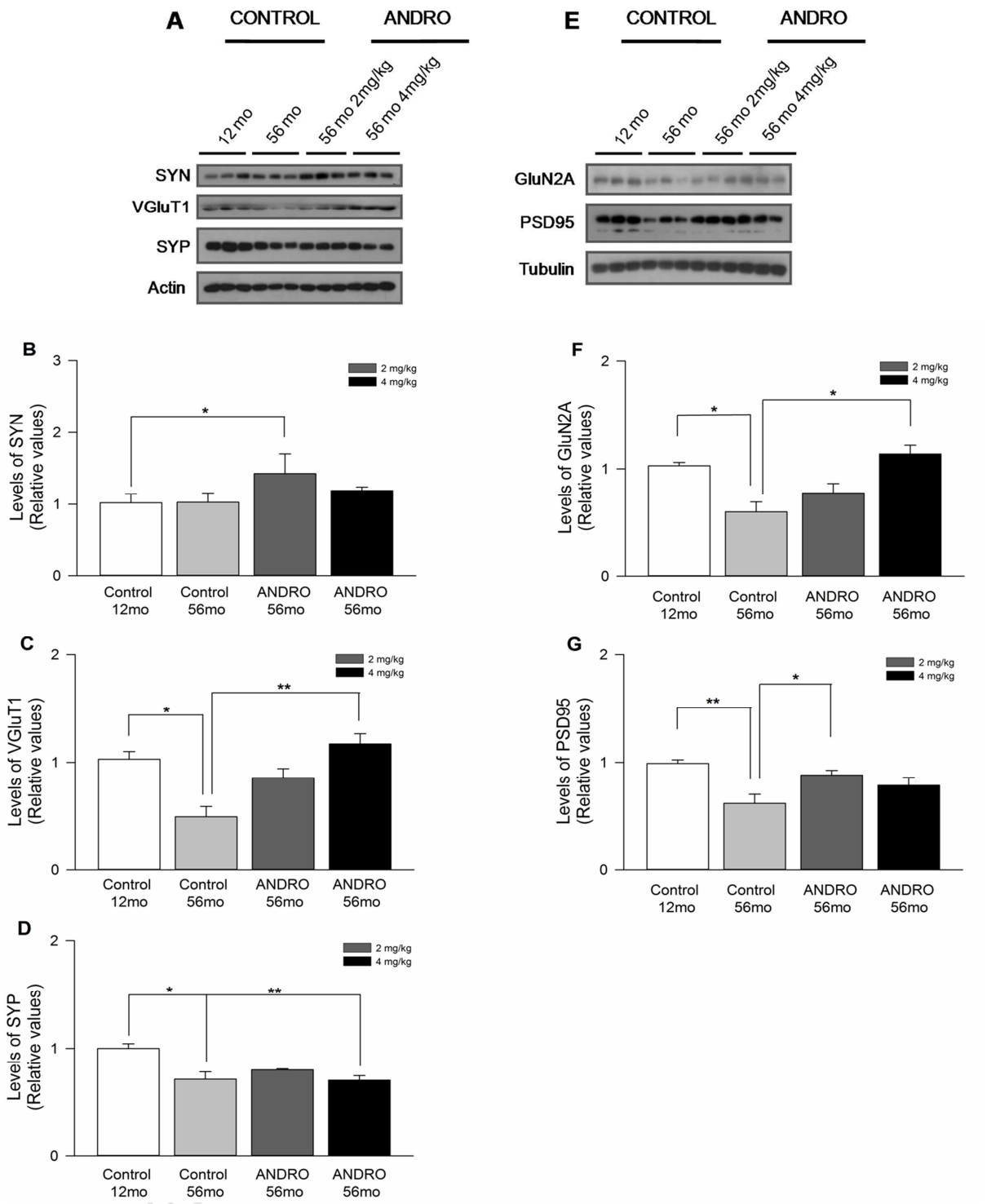


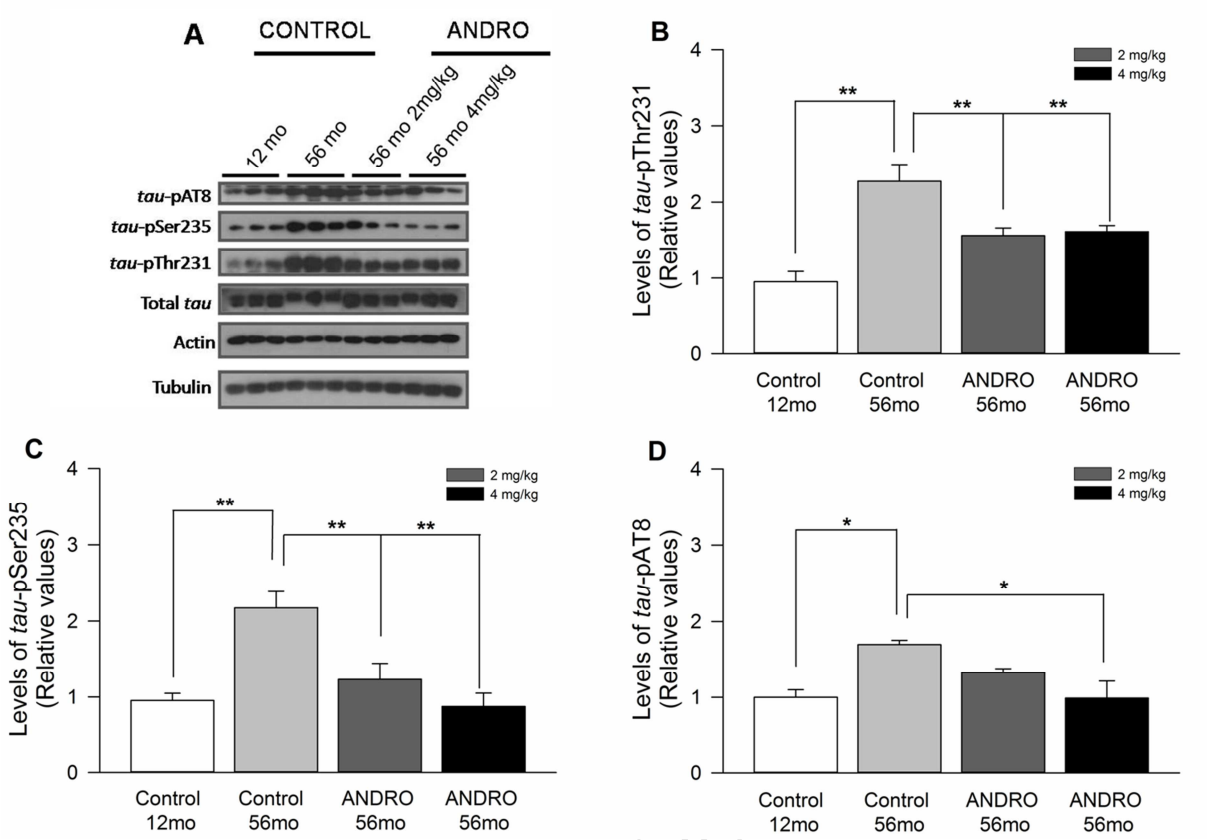


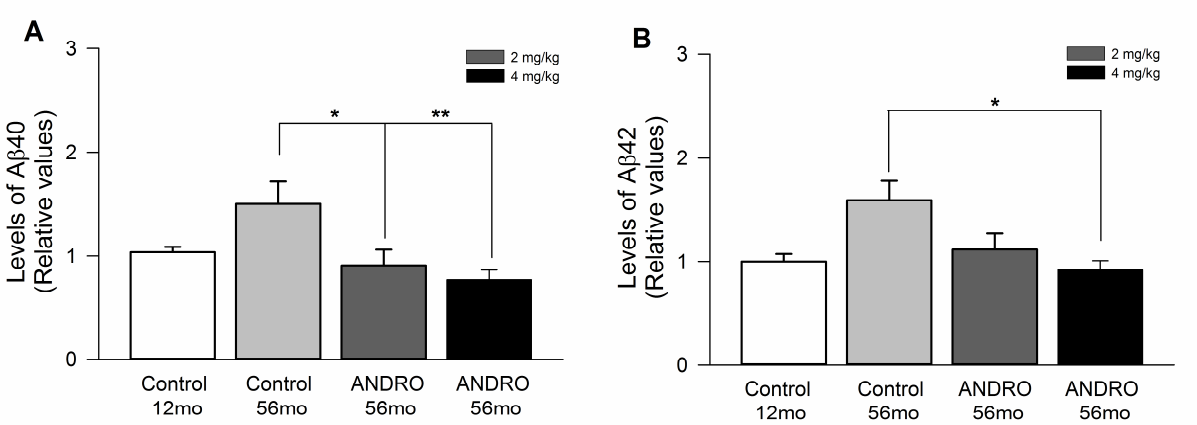


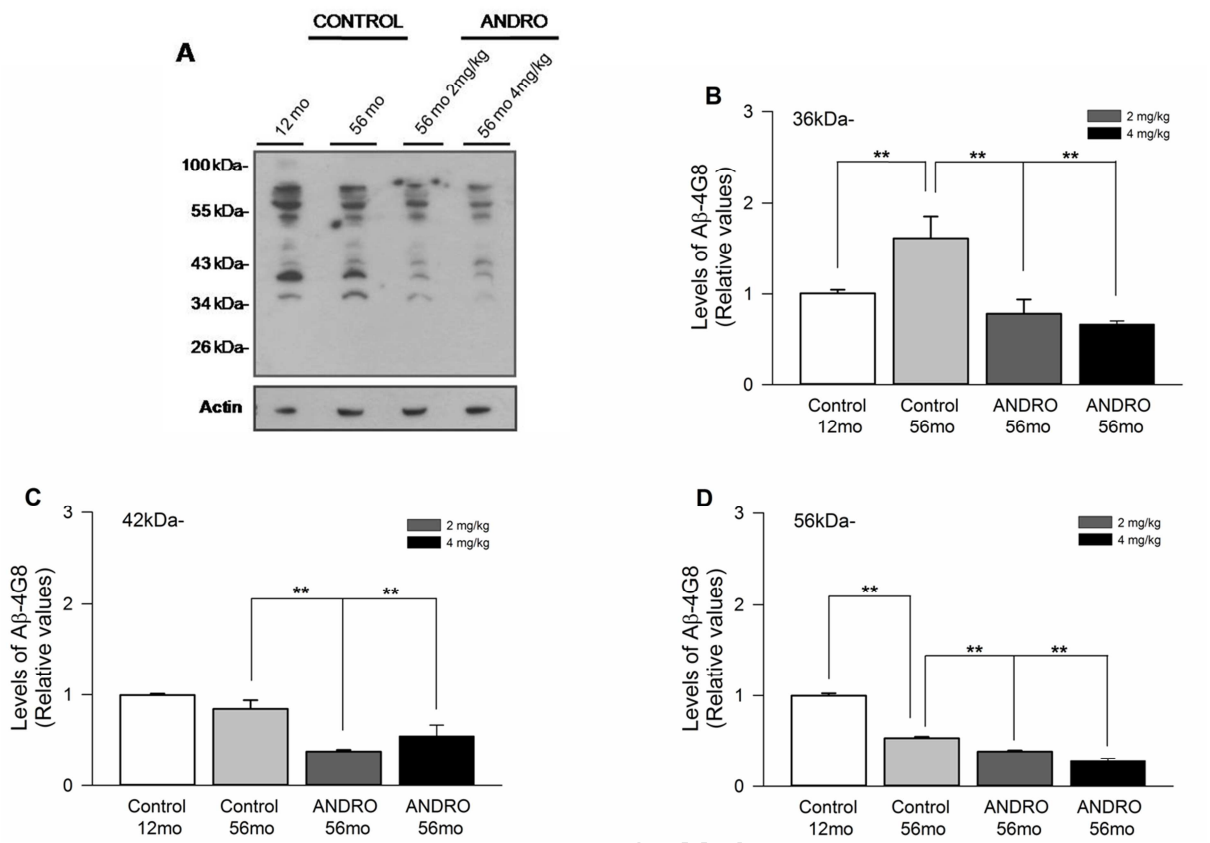

A
Control 12mo
Control 56mo
ANDRO 56mo
2mg/Kg
ANDRO 56mo
4mg/Kg
0.1 mV
10 ms
B
I-O slope
Slope (mV/s)
0.015
0.010
0.005
0.000
0
20
40
60
80
100
Stimulus
C
2 mg/Kg
4 mg/Kg
R1(mV) / R2(mV)
1.0
0.8
0.6
0.4
0.2
0.0
R1
R2
0.1 mV
5 ms
Control
12 mo
Control
56 mo
ANDRO
56 mo
ANDRO
56 mo

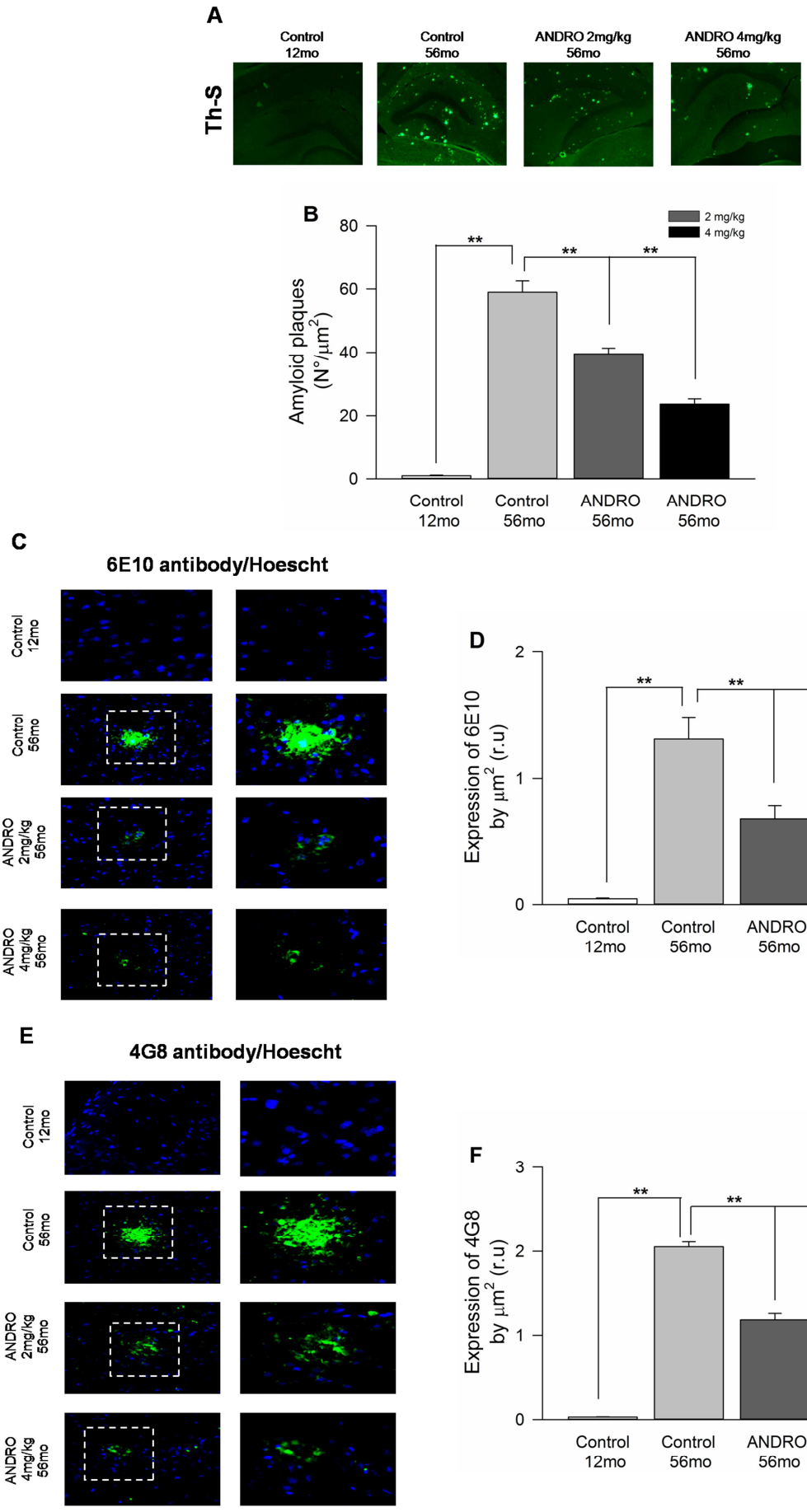

Control 12mo
1
2
Control 56mo
1
2
ANDRO 4mg/Kg
56mo
1
2
0.1 mV
10 ms
fEPSP slope (r.u.)
3.0
2.5
2.0
1.5
1.0
0.5
0
20
40
60
Time (min)
1
2
*
2 mg/Kg
4 mg/Kg
Control
12 mo
Control
56 mo
ANDRO
56 mo
ANDRO
56 mo


A
CONTROL
ANDRO
12 mo
56 mo
56 mo 2mg/kg
56 mo 4mg/kg
SYN
VGluT1
SYP
Actin
E
CONTROL
ANDRO
12 mo
56 mo
56 mo 2mg/kg
56 mo 4mg/kg
GluN2A
PSD95
Tubulin
B
Levels of SYN (Relative values)
2 mg/kg
4 mg/kg
Control 12mo
Control 56mo
ANDRO 56mo
ANDRO 56mo
C
Levels of VGluT1 (Relative values)
D
Levels of SYP (Relative values)
F
Levels of GluN2A (Relative values)
G
Levels of PSD95 (Relative values)








A
Control 12mo
Control 56mo
ANDRO 2mg/kg 56mo
ANDRO 4mg/kg 56mo
Th-S
B
2 mg/kg
4 mg/kg
Amyloid plaques (N°/µm²)
Control 12mo
Control 56mo
ANDRO 56mo
ANDRO 56mo
C
6E10 antibody/Hoescht
Control 12mo
Control 56mo
ANDRO 2mg/kg 56mo
ANDRO 4mg/kg 56mo
D
Expression of 6E10 by µm² (r.u)
E
4G8 antibody/Hoescht
F
Expression of 4G8 by µm² (r.u)

Highlights

- *O. degus* constitutes a natural model to study aging-related diseases such as AD.
- ANDRO treatment recovers spatial memory and learning performance.
- ANDRO treatment protects of postsynaptic proteins loss and recovers synaptic strength
- ANDRO treatment exerts a neuroprotective effect, including the reduction of phosphorylated tau protein and Aβ aggregates levels.