MEDICAL UNIVERSITY – PLEVEN
FACULTY OF MEDICINE
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EFFECTS OF MELATONERGIC COMPOUNDS ON ANIMAL MODELS OF NEURODEGENERATIVE DISEASES

ABSTRACT

of a dissertation submitted for the award of the educational and scientific degree

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Scientific Supervisors:
Prof. Milena Atanasova Atanasova-Radeva, PhD
Prof. Dr. Yana Dimitrova Chekalrova, MD, PhD

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The dissertation comprises 146 pages, including a literature review (45 pages), aims, objectives, materials and methods (13 pages), results (43 pages), discussion, conclusions, contributions, and a list of publications related to the dissertation (13 pages). The bibliography contains 167 references, all in Latin. The dissertation includes 31 figures and 2 tables.

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Scientific Jury:

1. Assoc. Prof. Daniela Marinova Pehlivanova, MD
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Materials related to the defense are published on the university's website: www.mu-pleven.bg

CONTENT

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List of Abbreviations Used

AD	Alzheimer's disease
sAD/LOAD	Sporadic Alzheimer's disease / Late-onset Alzheimer's disease
EOAD	Familial Alzheimer's disease / Early-onset Alzheimer's disease
CNS	Central nervous system
Aβ	Amyloid beta
aCSF	Artificial cerebrospinal fluid
Ago	Agomelatine
Apo E	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	Beta-secretase / Enzyme cleaving APP at the beta site
BBB	Blood-brain barrier
BL	basolateral nucleus of the amygdala
CA1, CA2, CA3	Three fields constituting the hippocampus (from "cornu ammonis" – the horn of Ammon Ra)
CTF	Carboxyl-terminal fragments
DG	Dentate gyrus
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze test
FDA	U.S. Food and Drug ministration
GABA	Gamma-aminobutyric acid
HPA axis	Hypothalamic-pituitary-adrenal axis
icv	Intracerebroventricular
IL-1β	Interleukin 1β
IWG-2	International Working Group 2
LDT	Light/dark box test
MAP	Microtubule associated protein
MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartate
NFT	Neurofibrillary tangles
OF	Open field test
PET	Positron emission tomography
Pir	Piriform cortex
PSEN	Presenilin
RAM	Eight-arm radial maze test
SPT	Sucrose preference test
TNF-α	Tumor necrosis factor alpha

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that results in impairment of memory, cognitive function, and activities of daily living. It is the most common form of dementia, mainly affecting the elderly, and is associated with loss of brain cells and impaired interneuronal communication.

There is no comprehensive treatment for AD, but therapies are available that can slow the progression of the disease or relieve some of the symptoms. Researchers' efforts are focused on regulating the balance of amyloid beta (Aβ) and tau protein metabolism, maintaining normal neurotransmitter levels, and therapies to sustain cognitive abilities and alleviate comorbid depression.

Experimental animal models play a crutial role in understanding the pathophysiology of AD. These models allow research in a controlled environment, enabling the causes and underlying molecular mechanisms characterizing the disease to be investigated, new drugs to be tested, and potential therapeutic approaches to be discovered before being tested in humans.

Agomelatine (Ago) is a novel type of antidepressant that is a selective agonist of melatonin MT1 and MT2 receptors and antagonist of 5-HT_{2C} receptors. A number of studies have found and demonstrated its abilities not only to favorably affect symptoms of mild and moderate depression, but also, like melatonin, to exert a chronobiotic effect and to influence circadian rhythm disturbances while improving cognitive function.

In view of the above and the established anti-inflammatory and neuroprotective effects of Ago in other models of neurodegenerative diseases, in two consecutive studies, we investigated the effect of the drug administered prophylactically (immediately after model induction) or palliatively (months after model induction) on behavioral, biochemical and histological abnormalities in two models of AD induced by streptozotocin (STZ) and amyloid-beta (Aβ) in male sexually mature rats.

Elucidating the efficacy of this melatonin analogue on certain aspects of pathogenesis in AD models would contribute to expanding its therapeutic application and further clinical studies.

Objective

To investigate the efficacy of the antidepressant agomelatine administered during the early and/or late stages of AD progression on the pathogenesis induced by intracerebroventricular infusion of toxins and associated with behavioral, biochemical, and cellular abnormalities in two animal models of AD.

Tasks

1. To make a model of sporadic Alzheimer's disease in male rats by intracerebroventricular infusion of streptozotocin.

2. To create a second model of sporadic Alzheimer's disease, by intracerebroventricular infusion of $A\beta_{1-42}$.

3. To validate the models by demonstrating cognitive and behavioural changes (tests reporting: memory, anxiety and depressive-like behaviour).

4. To investigate the effect of agomelatine on model-induced impairments in anxiety and worry levels, memory and cognitive functions.

5. To investigate biochemical markers of neurodegeneration and inflammation in frontal cortex and hippocampus in two rat models of AD, streptozotocin and amyloid.

6. To perform histological analysis of specific brain structures (piriform cortex, hippocampus, amygdala) from animals of the two models studied to assess morphological changes and neuronal loss.

Materials and methods

1. Experimental animals

Eighty-seven three-month-old male rats of the breeds Sprague Dawley (40 pcs.) and Wistar (47 pcs.), obtained from a licensed breeder - Vivarium at the Institute of Neurobiology, ADS, Sofia, were used for the experiments conducted to achieve the objectives of the thesis. Slivnitsa.

The experimental animals were adapted to the housing facilities at the Institute of Neurobiology (INB), Bulgarian Academy of Sciences (ADS) seven days before the AD induction procedures. Housing conditions were standardized: small groups of 3-4 animals in Plexiglas cages, 12/12-h light-dark cycle, with the light phase starting at 08:00 and the dark phase at 20:00; air temperature 21-22±2-3 °C; relative humidity 40-50% and free access to food and water, except for the time when the behavioural tests were performed.

The rules of the Ethics Committee of the INB, ADS (registration FWA 00003059 US Department of Health and Human Services) and the conditions of the Research Ethics Committee of the Medical University-Pleven were followed in the handling of the experimental animals. A permit for the use of animals in experiments was issued by the Bulgarian Food Safety Agency (No. 121, valid until 18.06.2020). Behavioral experiments were conducted in accordance with the seasonal circadian rhythms of rodents and were carried out during the autumn-winter period.

2. Induction of sAD

2.1. STZ-induced rat model of AD (Streptozotocin model)

Sprague-Dawley rats were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (20 mg/kg) injected intraperitoneally. They were then immobilized on a stereotaxic apparatus (Narishige Sci. Inst. Labs, Japan) and a longitudinal incision was made in the middle of the skin covering the skull and on the periosteum. Bilaterally through the calvarium, above the lateral ventricles, two small holes were made using a dental drill, with coordinates (AP= -0.8, L= ±1.5, H= 3.8) according to the atlas of Paxinos and Watson, (2006). Steel cannulas were implanted in each of the holes. A freshly prepared streptozotocin solution at a dose of 3mg/kg dissolved in arteficial cerebrospinal fluid (aCSF) was infused at a rate of 1µl/min for 3µl for each cannula (6µl total for both ventricles). The infusion was performed with a 28-gauge steel needle and a 10-µl Hamilton syringe (Hamilton®). The injection needle was left in place for five minutes to avoid back diffusion of the solution.

The STZ infusion procedure was performed three times within three days. After the third infusion, the scalp of the animals was closed with medical suture and the wound was treated with antiseptic antibiotic powder. Sham-veh groups were treated in the same manner, but were infused bilaterally with only an equivalent amount of aCSF. For their recovery after surgery, animals were injected with Ringer's solution (2 ml/100 g body weight/day, subcutaneously) for one week, received vitamins and were fed a moistened chow diet.

Ago injection was started ninety days after STZ infusion and continued for thirty days. A dose of 40 mg/kg, i.p. dissolved in hydroxyethylcellulose (1%) was used and injected daily at 16:00 h (two hours before the onset of the dark phase). This amount was shown to be effective in behavioral and neurobiological tests (Shingo et al., 2013; Tchekalarova et al., 2016). The Ago used was kindly provided by Servier Company, France.

The experimental animals were divided into three groups as follows:

- Sham (sham-operated) rats (sham-veh) treated with solvent alone (n=12);
- STZ-treated rats without subsequent Ago treatment (STZ-veh) (n= 11);
- STZ-infused rats treated with Ago (STZ-Ago) (n= 13).

The experimental design is shown in Figure 1.

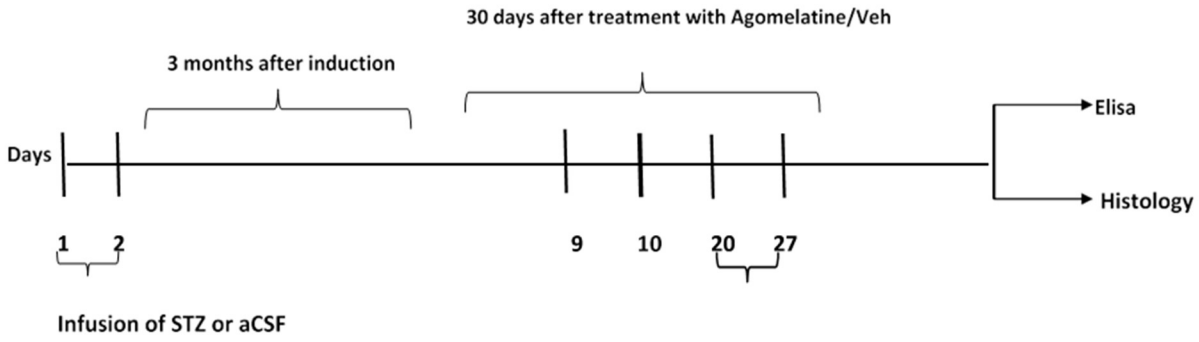


Figure 1. Schematic representation of the experimental protocol.

2.2. Aβ-induced rat model of AD (Amyloid model)

The anaesthesia and cannula amplantation procedure was similar to that used in the STZ model. Male Wistar rats were used. Amyloid $\beta_{1\text{-}42}$ (100 μg; Tocris Bioscience Bristol, UK) was dissolved in 100 μL aCSF and incubated at room temperature for one week prior to use to produce neurotoxic oligomers (Asadbegi et al., 2016). Infusion of $A\beta_{1\text{-}42}$ was performed under the same conditions as described for the STZ model. The same procedure was applied for the sham-operated group, except for the infusion of aCSF. The care of the operated animals, performed within one week, was similar to that described for the STZ model.

Experimental animals, we divided into two experimental groups, and a different Ago treatment protocol was applied to each group. In the first group, called "Experiment 1," treatment with Ago dissolved in 1% hydroxyethylcellulose, at a dose of 40 mg/kg, was started in parallel with icv $A\beta_{1\text{-}42}$. In the second group, "Experiment 2", Ago treatment was started 30 days after icv of $A\beta_{1\text{-}42}$.

In both experiments, Ago was injected i.p. about two hours before the onset of the dark phase for 30 days, in a similar manner to the STZ-induced model.

In Experiment 1, the following groups were studied:

- rats undergoing surgery, infused with solvent alone (sham-veh) (n=8);
- rats infused with Aβ1-42 and no subsequent Ago treatment (Aβ-veh) (n=10);
- Aβ1-42-infused rats treated with Ago (Aβ-Ago1) (n=9).

They were used for behavioral tests, histological and biochemical analysis.

In Experiment 2, the groups were analogous, each consisting of 6-8 numbers of experimental animals: sham-veh group; Aβ-veh and Aβ-Ago2.

Brain structures from Experiment 1 were used for biochemical and histological analysis, and those from Experiment 2 were examined biochemically only.

The detailed study design is schematically presented in Figure 2.

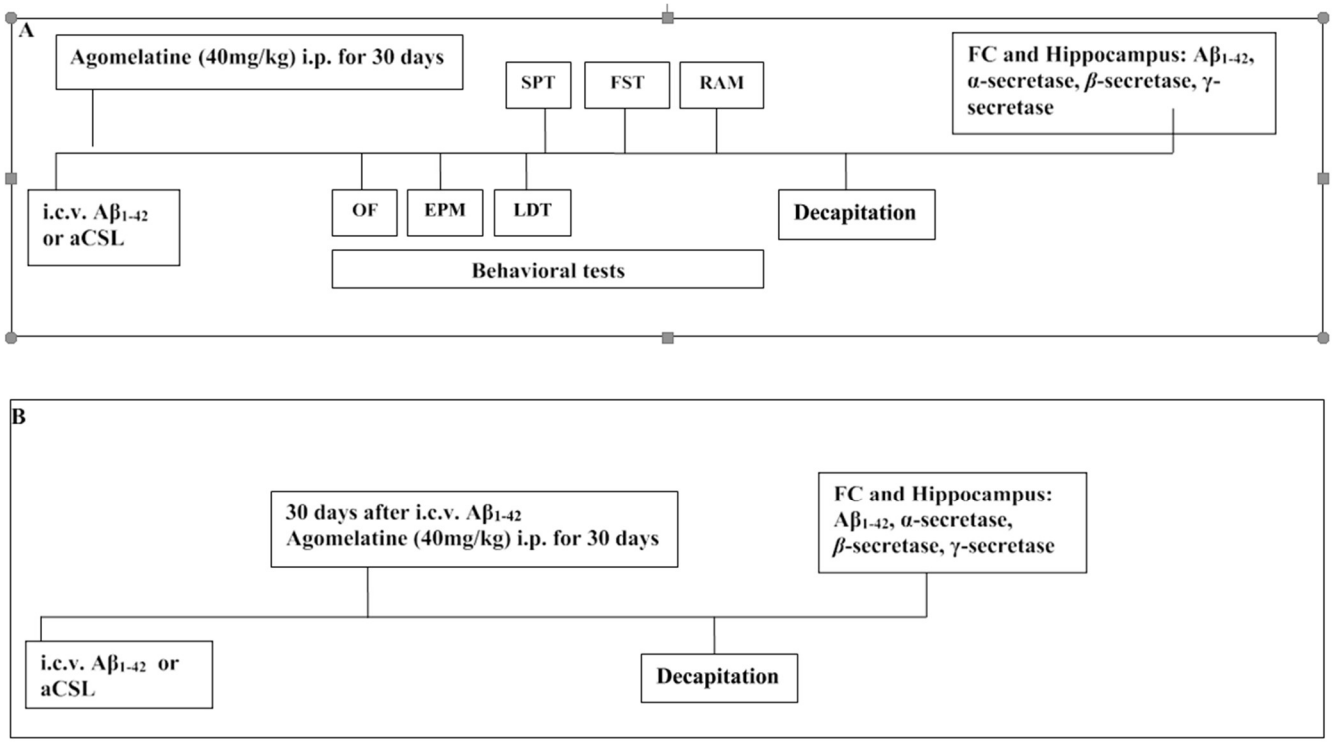


Figure 2. Schematic representation of the experimental protocol.

3. Behavioural tests

The effect of chronic Ago treatment on behavioral changes and spatial memory was assessed using a battery of tests that were performed between 10:00 am and 12:00 pm in a separate soundproof room, under artificial diffuse light, where rats were housed for at least 30 min before each test.

3.1. Open field(OF) test

The OF test was conducted in a polystyrene open box (100/100/60 cm) for 5 min. The rat was carefully placed in the central area of the box and the total distance traveled (cm), vertical activity (number of hind paw rears), and time spent in the center of the box (sec) were recorded.

3.2. Elevated cross maze (EPM) test

The apparatus, made of black wood, has two open arms (50/10 cm) perpendicular to two closed arms (50/10/50 cm) and a central open platform (10/10 cm). Each rat was placed in the center of the maze, facing one of the two open arms. The following parameters were measured: total distance traveled (cm), number of entries, and time spent in the open arms in 5 min.

3.3. Light-Dark Field Test (LDT)

The LDT test was performed in a grey polystyrene apparatus consisting of one indoor (dark) (25 cm 25 cm 40 cm) and one outdoor (light) (25 /50 / 40 cm) compartment connected by a 7 cm open area. A light bulb (80 lx) was mounted on top of the open compartment. Each rat was carefully placed in this open area and the following parameters were measured: total time spent in the light compartment (sec), number of transitions, and latency period to enter the dark area for 5 min.

3.4. Test to investigate preference for sweet solutions (SPT)

Each experimental animal was placed in a separate cage. The habituation test without measurement was performed for 5 days with two identical graduated 100 ml bottles, one filled with tap water and the other with 1% saccharin solution. Preference for the sweet solution was assessed by measuring the percentage ratio of the volume of sweet solution to the total volume of liquid consumed during the light and dark phases, respectively.

3.5. Forced swimming test

The forced swim test (FST) was performed in a plastic, transparent cylinder (60 cm height, 45 cm diameter) filled with water (23°C), up to 30 cm, for 5 min, according to an adapted protocol of Porsolt et al. (1979). The rat's movement just to maintain the head above the water surface was recorded as time of immobility (sec) and recorded manually.

3.6. Spatial memory study using an eight-arm radial arm maze (RAM)

Prior to the test, all rats were subjected to a diet to reduce body weight by up to 15% of baseline for at least one week. A three-day habituation pre-test was performed in the maze with sweet food pellets placed along all arms. Rats that did not learn to eat more than 70% of the pellets in 15 min were excluded from the study. The test consisted of one session per day conducted for 5 consecutive days. A sweet pellet was placed at the end of each arm. The test animal was placed in the central area of the apparatus. The end of the session was considered when all pellets were eaten, and after the 10th minute if the criterion was not met. Re-entry into an arm from which the food pellet had been removed and was therefore already empty was recorded as a working memory error.

4. Biochemical parameters measured by ELISA.

4.1. Measurement of Aβ, TNF-α and IL-1β by ELISA.

After decapitating the animals, the hippocampus and frontal cortex were isolated on ice and stored at -20 °C until biochemical assays were performed. Tissue samples were homogenized in 10 ml/g tissue cold buffer containing 10 mM Tris HCl (pH 7.6), 1 mM EGTA, 50 mM NaF, 1 mM EDTA and 1 mM PMSF.

The three protein markers were analyzed using ELISA kits purchased respectively: for Aβ from AnaSpec Company; for TNF-α and IL-1β from Invitrogen. TNF-α and IL-1β measurements were performed after additional centrifugation at 12,000g, 4°C, for 10min of tissue homogenate. The values of the tested markers were measured and presented in pg/ml.

After homogenization, the protein concentration in each sample was measured by the Bradford assay. Amyloid β1-42 was measured with a kit (AnaSpec, SensoLyte®, cat. no. AS- 55,553).

After measuring the amyloid concentration in the samples, we divided this value by the protein content value to obtain the amount of β-amyloid relative to 1 mg protein. Quantities were presented as pg/mg protein.

4.2 Western blot for tau protein measurement

The hippocampal and frontal cortex homogenates were centrifuged at 12,000 g, 4°C, for 10 min. The protein concentration in the supernatant was determined by the Bradford method. Equal amounts (20 mg/well) of each sample were applied to a 10% SDS polyacrylamide gel. To each gel, one of the samples served as a standard (brain homogenate at a concentration of 20 mg/ml). After electrophoretic separation, the proteins from the gel were transferred onto a nitrocellulose membrane

and blocked with 3% bovine serum albumin in TBS-0.05% Tween, for 3 h. The membrane was incubated with primary rabbit anti-tau (pSer356) phospho-specific antibody (MBL International corporation) diluted 1:1000, for 3 h at room temperature or overnight at 4 °C. This was followed by washing three times with TBS-Tween and further incubation in secondary biotinylated anti-rabbit IgG antibody (Vector laboratories) diluted 1:1000. After washing three times in TBS-Tween, the membrane was incubated in a solution of avidin and biotin, then calibrated in PBS (pH 7.2), then in a solution of DAB-peroxidase substrate at room temperature for 15 min. After the appearance of color bands of appropriate density, the membrane was rinsed in PBS and air dried. Blots were scanned and analyzed with ImageJ software (V 1.51u). Results were reported in relative units, AU (arbitrary units) and normalized to mg/protein.

4.3. Measurement of α-secretase, β-secretase and γ-secretase by ELISA

The β-secretase and γ-secretase content was analysed according to the procedure described by the manufacturer (Elabscience Cat. No. EL-R0102 and Cat. No. EL-R2489). The amounts obtained from each sample, expressed in pg/ml, were divided by the measured protein content. Samples for α-secretase quantification were also assayed by ELISA (BlueGene, Cat. No. E02A0727) and the amount of α-secretase was expressed in pg/mg protein.

5. Histology

Rats were deeply anesthetized with urethane (1500 mg/kg, i.p.) (n=5 per group) from Experiment 1 and transcardially perfused first with 0.05 M phosphate-buffered saline (PBS), followed by 4% para-formaldehyde in 0.1 M phosphate buffer (PB), pH 7.36. After perfusion, isolated brains were postfixed in the same fixative overnight at 4◦C. The rat brain tissue blocks were then washed in tap water and then in distilled water. After dehydration, they were embedded in paraffin, cut with a microtome to 6 μm thickness, and mounted on chrome gelatinized glass slides. Brain sections were deparaffinized with xylene and ethanol and stained with hematoxylin and eosin. Cell density was measured using Nikon's NIS Elements digital imaging software as previously described (Tchekalarova et al., 2017), and quantitative analysis of cell density was performed by determining the number of stained cells in the selected brain structure.

6. Statistical analysis

The experimental data were statistically processed using SigmaStat® 11.0 and GraphPad Prizm 7. The presence of a significant difference between groups was verified by a one-factor ANOVA followed by a Bonferroni post hoc test. A two-factor ANOVA was used for the RAM test data. If the data were not normally distributed, ANOVA was used for nonparametric data (Kruskal-Wallis for ranks) followed by Mann-Whitney U test. Results were presented as mean ± SEM. Statistically significant differences were accepted at $p<0.05$. The magnitude of differences found was expressed as effect size (η^2 for ANOVA and Cohen's d for pairwise comparisons) in symmetrically distributed data.

Results

1. Examining the effects of chronic agomelatonin treatment on emotional and cognitive deficits in a model of AD

1.1. Investigation of anxiety and motor activity levels

1.1.1. Elevated plus maze (EPM) test

Decreased frequency of entry into the open arms, which are an aversive zone for rodents, dwell time and total distance travelled within them have been accepted as markers of increased anxiety.

Streptozotocin model: compared to controls, STZ-veh rats exhibited increased anxiety and traveled a shorter distance (Kruskal-Wallis test: H= 12.7, p= 0.002 η^2_p= 0.53), spent less time (one-factor ANOVA: $F_{2,21}$ = 9.5, p= 0.002, Cohen's d= 0.9 η^2_p= 0.33) and made fewer entries (one-factor ANOVA: $F_{2,21}$= 3.9, p= 0.039, Cohen's d= 0.9 η^2_p= 0.33) into the open arms of the maze (Fig. 3). Chronic treatment with Ago significantly ameliorated STZ-induced behavioral changes by alleviating anxiety behavior as expressed by distance traveled ($p<0.001$), dwell time ($p<0.001$; d= 2.1), and number of entries (p= 0.003; d= 1.4) in the open arms compared to rats in the STZ-veh group.

Amyloid model: intracerebroventricular infusion of $A\beta_{1-42}$ induced an increased level of anxiety of the experimental animals, which was clearly reported by all the listed indices (Kruskal-Wallis test: H= 15.24; Mann-Whitney: U=10 p= 0.0014; U= 4, p< 0.0005). Statistically significant differences were reported for total distance travelled (Fig. 4B), as in the STZ model. Post hoc analysis showed that the Aβ-veh group had a significantly reduced number of entries and visits to the open arms (Fig. 4A and C). Ago treatment alleviated icv$A\beta_{1-42}$-induced increased anxiety (number of entries: p = 0.03; time: p = 0.002 in the open arms).

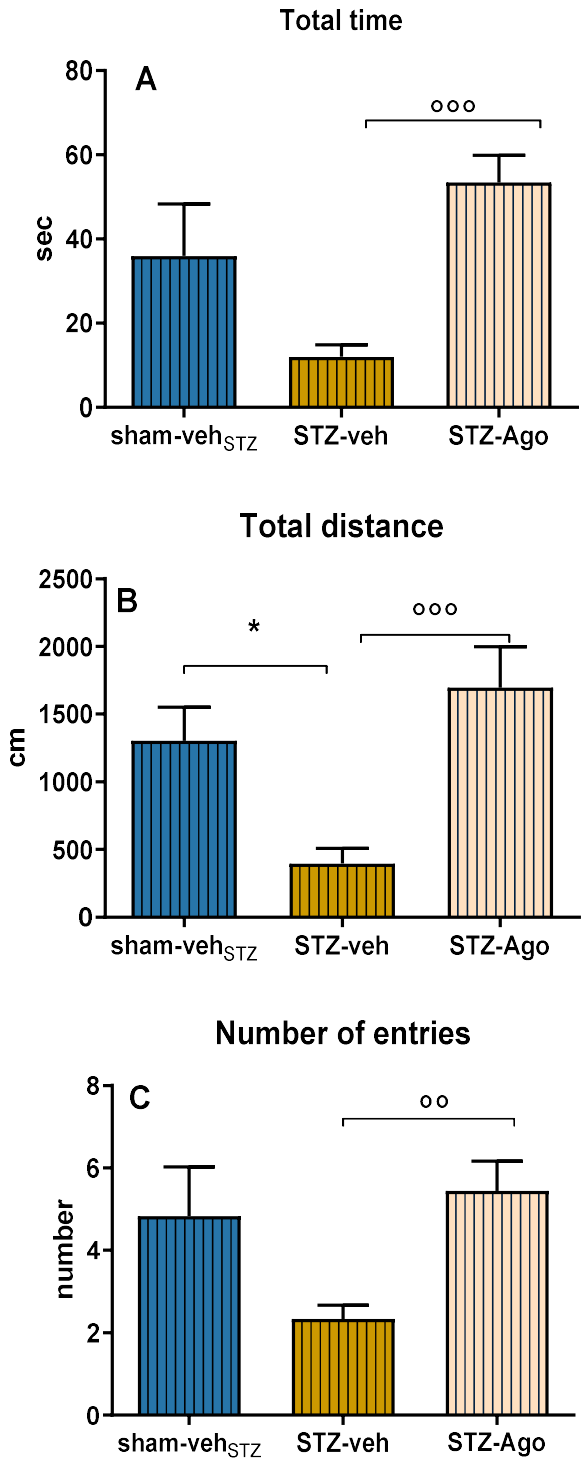


*Figure 3. Effect of chronic Ago treatment, in the STZ model of AD, on the dwell time (sec), distance traveled (cm), and number of visits to the open arms of the elevated cross maze. Data are presented as $\bar{x} \pm$ S.E.M. *comparison of STZ-veh with sham-veh; comparison of STZ-Ago with STZ-veh.*

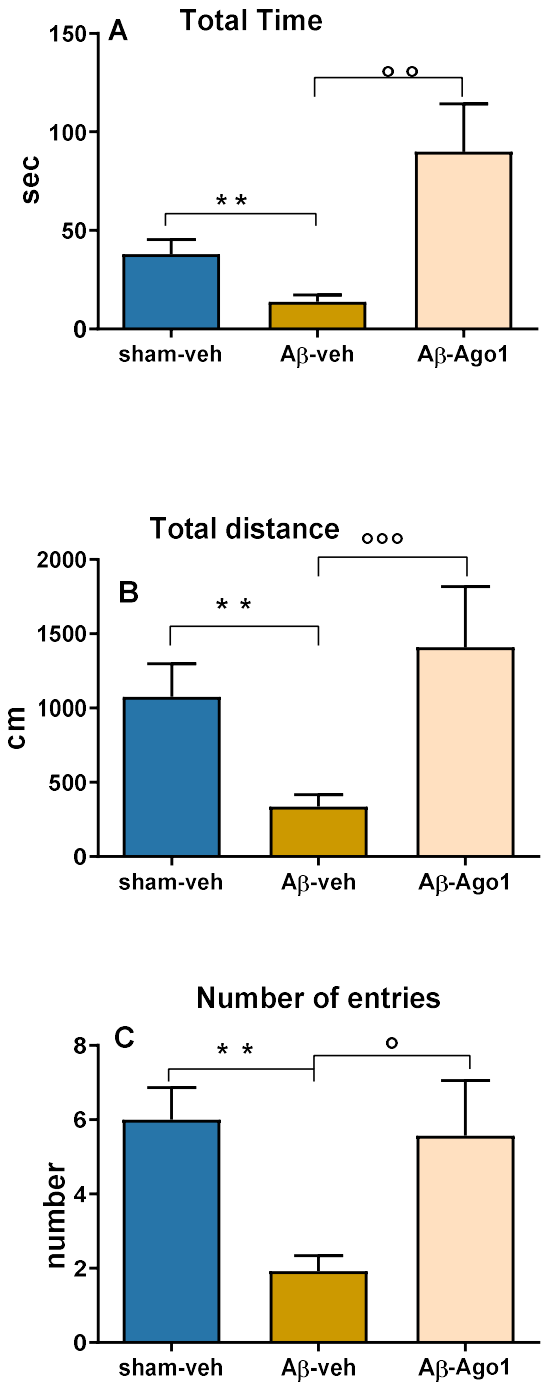


*Figure 4. Figure 4. Effect of chronic Ago treatment, in the $A\beta_{1\text{-}42}$ model of AD, on dwell time (sec) (A), distance traveled (cm) (B), and number of entries (C) into the open arms in the EPM test. Data are presented as $\bar{x} \pm$ S.E.M. *comparison of Aβ-veh with sham-veh; comparison of Aβ-Ago with Aβ-veh.*

1.1.2. Open field (OF) test

The central area of the OF test apparatus is thought to be perceived as an aversive area by rats and other rodents. Longer study periods and longer stays in the central zone indicate reduced levels of anxiety.

Streptozotocin model: The distance traveled by the STZ-veh group was significantly less than that of the control group. The distance traveled by STZ-Ago exceeded that traveled by sham-operated animals (Figure 5A). The group of animals with induced AD spent the shortest time in the central zone compared to controls and agomelatine-treated animals, an indication that their anxiety levels were higher, possibly due to STZ intoxication. The reported difference was statistically significant only between STZ-veh and STZ-Ago. (Figure 5B).

Amyloid model: Motor activity, as expressed by total distance travelled, was not affected by the icv infusion of Aβ (Figure 6A). However, changes in exploratory activity, such as a decrease in the number of hind paw rearings ($F_{2, 31}$= 9.284; p= 0.0007), and also in anxiety levels, increasing the time spent in the central part of the apparatus ($F_{2, 31}$= 15.11; p< 0.0001) were recorded. Comparison between groups showed that Aβ-veh had lower vertical activity (t= 4.346, df= 23) compared to the sham-veh group (Figure 6, B) and shorter time spent in the aversive central area of the apparatus (t= 3.806, df= 2, p= 0.001) (Figure 6C) compared to sham-operated animals. Chronic Ago treatment restored to control level the icv$A\beta_{1-42}$-induced attenuated behavioral response associated with the number of hindpaw rests (t= 3.479, df= 20) and time spent in the center compared to the Aβ-veh group (t= 5.505, df= 19, p< 0.0001).

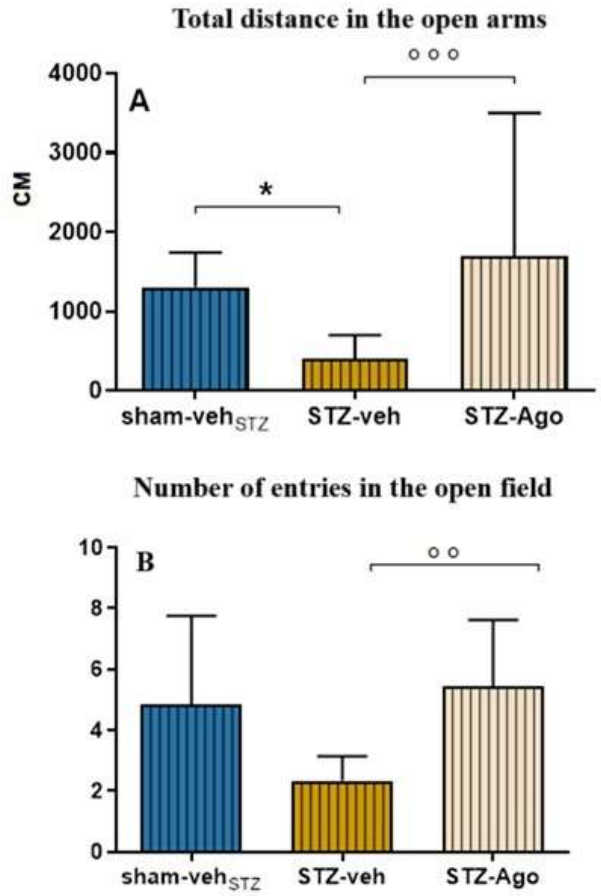


*Figure 5. Effect of chronic agomelatine treatment (Ago), on total distance (cm) (A) and open area visits (B) in the OF test. Data are presented as $\bar{x} \pm$ S.E.M. *comparison of STZ-veh with sham-veh; comparison of STZ-Ago with STZ-veh.*

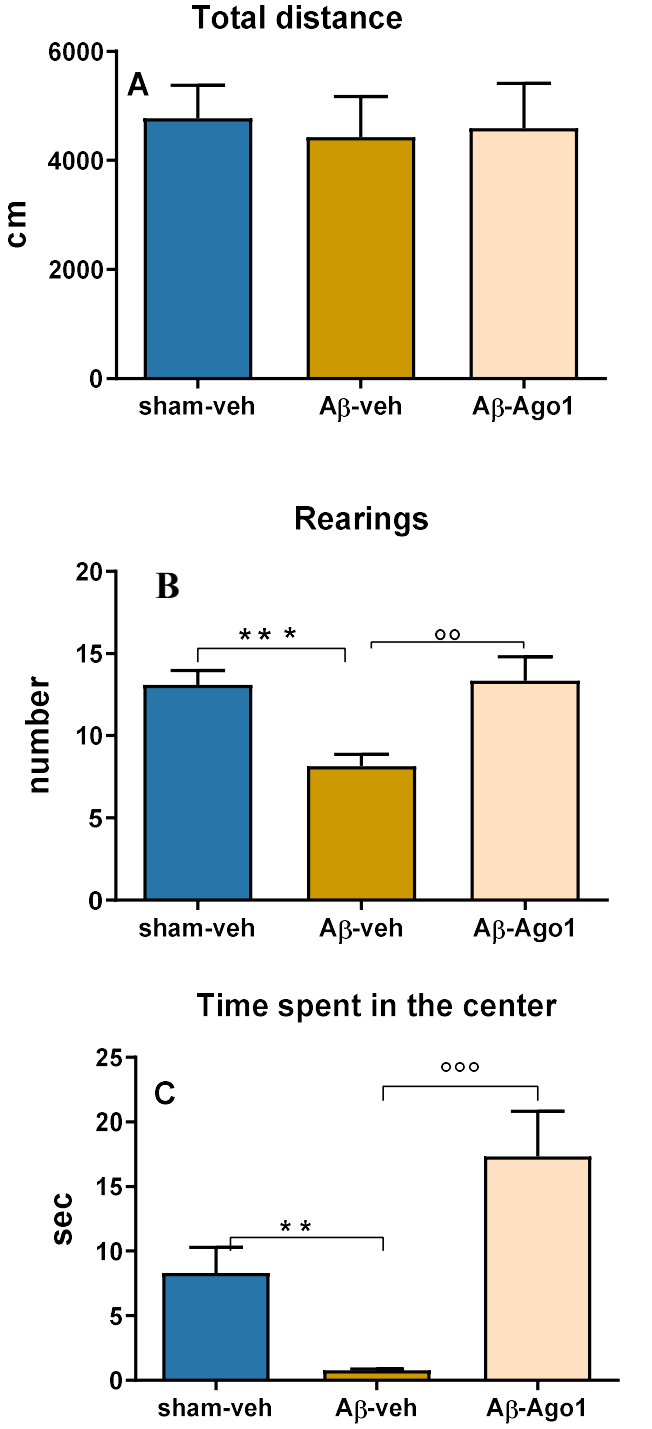
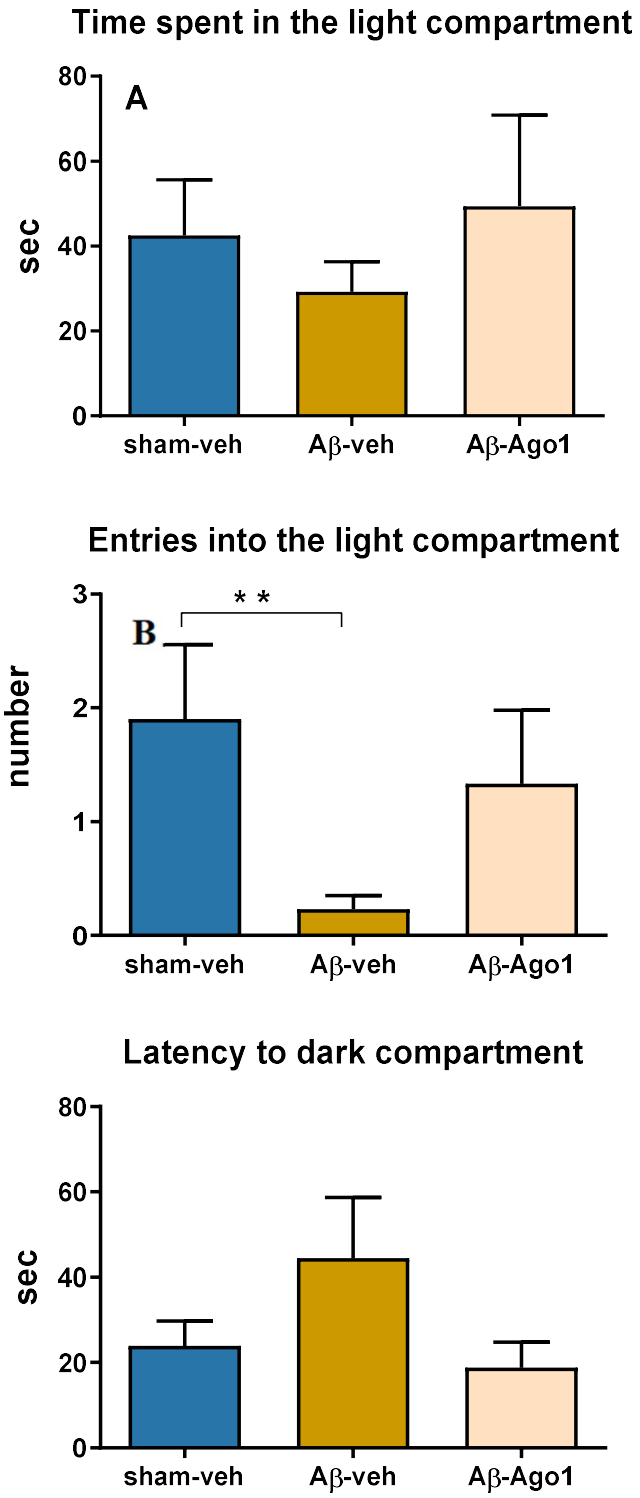


*Figure 6. Influence of chronic Ago on total distance in cm (A), rearings (B) and time spent in the center (C) in the open field test. Data are presented as $\bar{x} \pm$ S.E.M. * comparison of Aβ-veh with sham-veh;° comparison of Aβ-Ago with Aβ-veh.*



1.1.3. Light/dark field test-LDT (light-dark test)

The lighted compartment is also considered an aversive area in this method and the time spent there, the number of entries into the dark compartment and the time to move into it are three indicators by which we report the level of anxiety in the experimental animals. The method was applied only to the Amyloid model animals, in Experiment 1, to examine another aspect of anxiety related to the exploratory instinct in animals. Similar to the other two tests OF and EPM, we observed an increase in the level of anxiety in the rats of the Aβ-veh group, which was induced by ivcA$\beta_{1\text{-}42}$.

Although no statistically significant difference was reached in the time spent in the light compartment ($H = 0.405$, $p = 0.817$) and the latency to move to the dark compartment ($H = 4.448$, $p = 0.108$), there was a trend for a change in the values of these two parameters, namely: a shorter stay in the aversive zone and an increase in the time required to move to it, in the Aβ-veh group compared to the sham-veh and Aβ-Ago groups. From the results presented in Figure 7, we see that, nevertheless, the number of transitions from light to dark was significantly different among the three groups ($H= 5.863$, $p= 0.05$), and was noticeably reduced in the Aβ-veh group ($p= 0.01$).

*Figure 7. Effect of chronic Ago treatment on time spent in the light compartment (sec) (A), number of transitions (B), and dark compartment entry latency (C). Data are presented as $\bar{x}$ ± S.E.M. **p<0.01 *comparison of Aβ-veh with sham-veh;° comparison of Aβ-Ago with Aβ-veh.*

1.2. Screening for comorbid depression associated with AD.

1.2.1. Sweet solution preference test.

Anhedonia, associated with a lower tendency to consume sweetened solution compared to drinking water, was accepted as a marker of depressive-like behaviour. Preference for sweet solutions was negatively affected by infusion of both toxins by which we induced AD in both experimental models.

Streptozotocin model: STZ administration significantly reduced the volume of sweet solution ingested relative to the total volume of fluid ingested. Treatment with the melatonin analogue, Ago, after STZ infusion resulted in a statistically significant increase in sweet solution consumption (one-factor ANOVA analysis: $F_{2, 22}= 40.3$, $p< 0.001$). Against this parameter we can judge that an alleviation of the anhedonia condition induced by STZ occurred (Figure 8) ($t= 8.764$, $df= 14$, $p< 0.0001$).

Amyloid model: after infusion of icvAβ1-42 (one-factor ANOVA analysis: $F_{2,23}=32.330$, $p<0.0001$), preference for sweet solutions was significantly reduced. There was a significant reduction in consumption of sweetened solution by the Aβ-veh group compared to the sham-veh group ($p<0.0001$). Agomelatine was able to correct the depression-like response in the Aβ-Ago group ($p= 0.0001$), respectively, compared to the Aβ-veh group and restore the affinity for sweetened solution to control levels ($t= 6.439$, $df= 17$, $p< 0.0001$) (Figure 9).

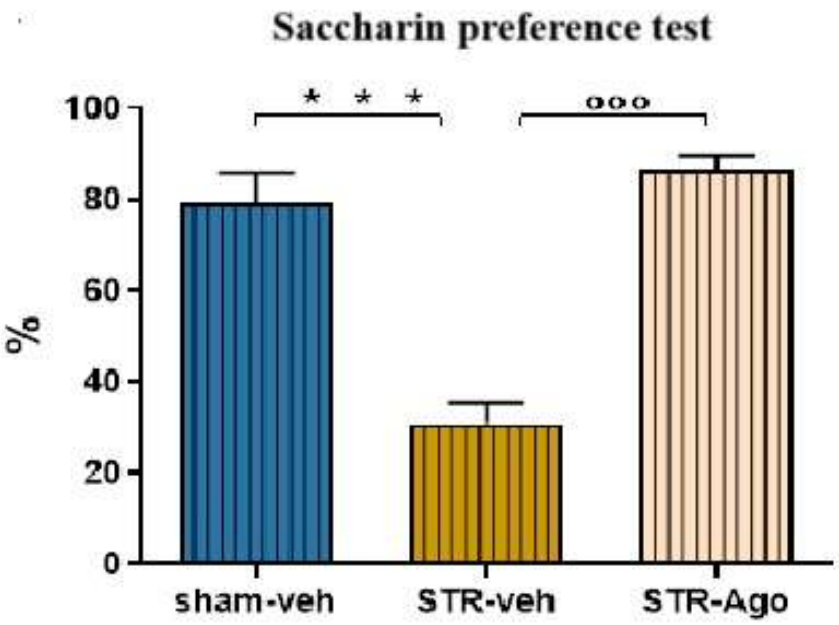


*Figure 8. Effect of chronic Ago treatment, in the icv-STZ model of AD, on preference for sweet solution (%). Data are presented as $\bar{x} \pm$ S.E.M. *comparison of STZ-veh with sham-veh; comparison of STZ-Ago with STZ-veh.*

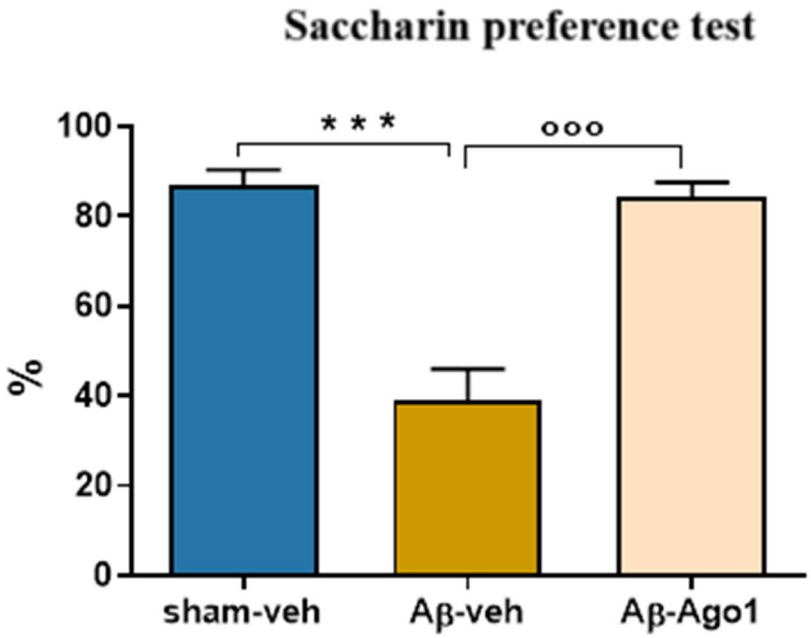


*Figure 9. Effect of chronic Ago treatment, in the icvAβ model of AD, on preference for sweet solutions (%). Data are presented as $\bar{x} \pm$ S.E.M. * comparison of Aβ-veh with sham-veh;° comparison of Aβ-Ago with Aβ-veh.*

1.2.2. Forced swimming test

Streptozotocin model: Increased immobility when placed in the water container is considered as a marker of despair-related behavior in FST. The results of the forced swimming test analysed with the experimental animals of the STZ model showed no significant difference in the behaviour of the animals of the three groups (Mann Whitney test, p= 0.9703). In Figure 10, it is clearly seen that the time spent in immobility was similar in the controls, the rats with induced AD and those with Ago treatment applied.

Amyloid model: One-way ANOVA analysis revealed (Figure 11) a significant difference in immobility time between sham-veh and Aβ-veh groups ($F_{2,23}$= 5.831, p= 0.009). Post hoc analysis confirmed that it was significantly increased in the Aβ-veh group compared to the sham-veh group (p= 0.016). The administered Ago treatment corrected the depressive-like behavior induced by icv$A\beta_{1-42}$ infusion.

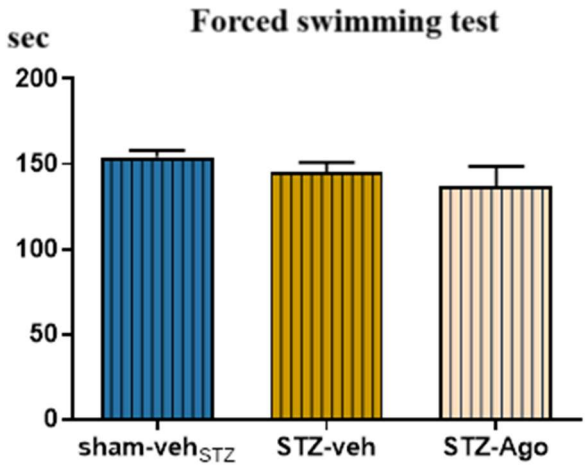


Figure 8. The forced swimming test reported no statistically significant differences in the behavior of the rats from the three groups of the STZ model.

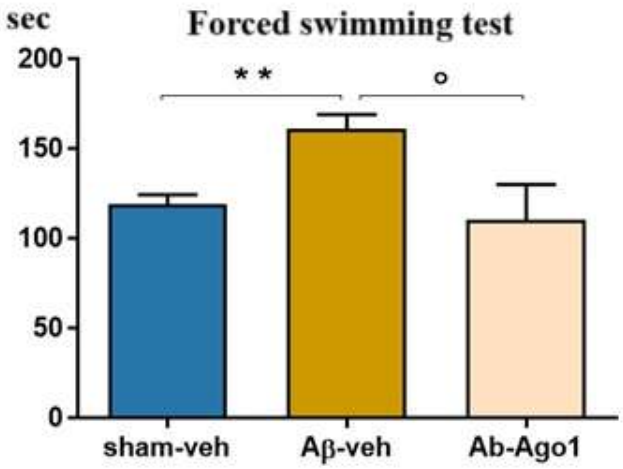


Figure 9. Aβ-veh rats exhibited depressive-like behavior, staying immobile longer during FST compared to the sham-veh group (p=0.001). Ago treatment exerted an antidepressant effect and significantly shortened the immobility time (t-test, p= 0.0121).

1.3. Test for hippocampus-dependent spatial memory - radial eight-arm maze - RAM

The lack of a decreasing trend in the number of spatial memory errors and task criterion time in each successive session are considered indicators of hippocampus-dependent spatial memory impairments. Sham-operated animals (in both experimental models) showed clearly how on each subsequent day of the experiment the time required to perform the task decreased, and also, made a decreasing number of errors.

Streptozotocin model: Two-factor ANOVA (factors were execution time and error count) analysis reported that the frequency of working memory errors was affected relative to the experimental group ($F_{2,104}$= 10.9, $p < 0.001$). We also reported a correlation between animal group and required execution time ($F_{8,104}$= 2.6, $p< 0.012$), which showed that STZicv infusion significantly impaired spatial memory. The post hoc test showed that the STZ-veh group required longer time to perform the task compared to the other two groups, respectively, relative to the sham-veh (4^{th} and 5^{th} session: $p< 0.001$ and $p= 0.009$, respectively) and relative to the STZ-Ago group (4th and 5th session: $p< 0.001$ and $p= 0.006$, respectively, $\eta^2_p= 0.51$) (Figure 12).

Amyloid model: In the second experimental model induced by Aβ infusion in both toxin-treated groups (Aβ-veh and Aβ-Ago), we noticed impairments in spatial memory, but found no significant change in the number of errors made over the time course of the test (Treatment effect: $F_{2,162}$= 26.863, $p< 0.001$; Session day: $F_{4,162}$= 5.022, $p< 0.001$). Post hoc analysis confirmed that the Aβ-veh group showed more working memory errors during the second ($p< 0.001$), fourth ($p= 0.007$) and fifth ($p< 0.001$) sessions compared to the sham-operated group (Figure 13). Chronic Ago treatment did not improve spatial memory in the Aβ group (second session $p= 0.012$), fourth ($p= 0.001$) and fifth ($p= 0.006$) session compared to the sham-veh group. The sham-veh group showed a tendency to perform the memory task in a shorter period with each session, whereas the two Aβ groups (Aβ-veh and Aβ-Ago) failed to shorten the performance time (Treatment effect: F2,162= 8.496, $p<0.001$; session day: F4,162= 10.420, $p<0.001$; correlation of treatment with Ago versus session day: F8,162= 8.953, $p<0.001$). Post hoc analysis showed that the Aβ groups (Aβ-veh and Aβ-Ago1) needed significantly more time on days four ($p = 0.023$ Aβ-veh compared to sham-veh group; $p = 0.018$ Aβ-Ago) and five of the session ($p < 0.001$ Aβ-veh; $p = 0.016$ Aβ-Ago compared to sham-veh group).

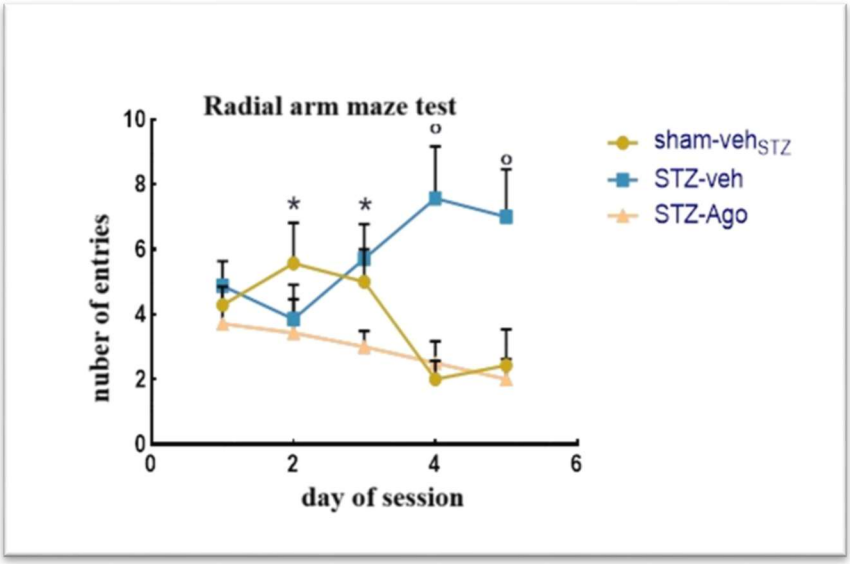


*Figure 10. Effect of chronic treatment with Aggo on working memory errors in the radial maze test. Data are presented as $\bar{x}$ ± S.E.M. *comparison of STZ-veh with sham-veh; comparison of STZ-Ago with STZ-veh.*

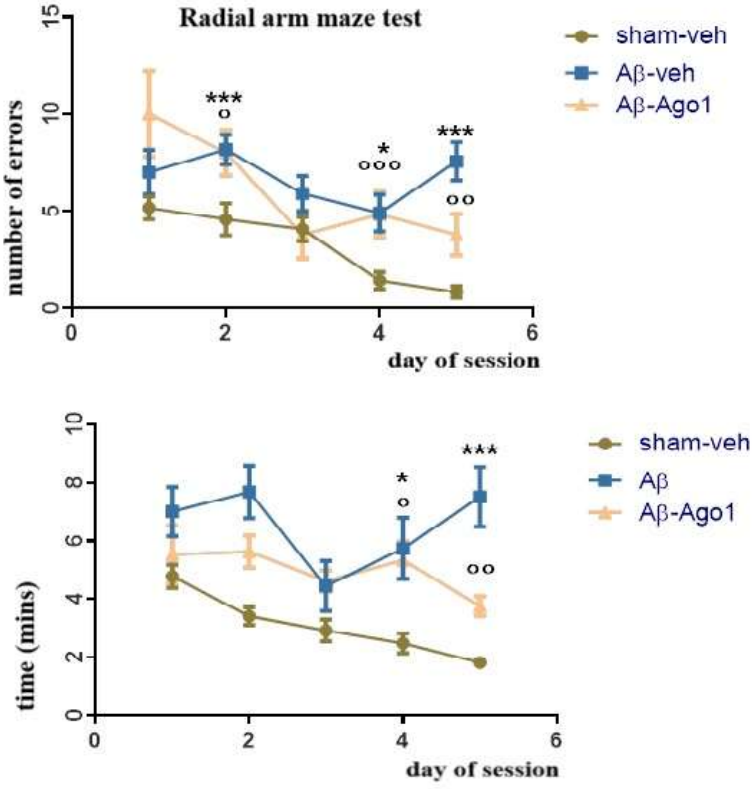


*Figure 11. Effect of chronic treatment with Aggo on working memory errors in the radial maze test. Data are presented as $\bar{x}$ ± S.E.M. **p= 0.007 day 4, ***p< 0.001 day 5, Aβ-veh group compared with sham-veh group (A); *p= 0.023 4th day Aβ-veh group compared to sham-veh group, *p=0.018 4th day Aβ-Ago1 compared to sham-veh group, ***p<0.001 5th day Aβ-veh group compared to sham-veh group, *p=0.016 5th day Aβ-Ago1 compared to sham-veh group (B). (Mixed ANOVA); *comparison of Aβ-veh with sham-veh;° comparison of Aβ-Ago with Aβ-veh***P < 0.001) 2nd day.*

2. Effect of agomelatine treatment on the levels of biochemical parameters

2.1. Effects of agomelatine on Aβ levels in frontal cortex /FC/ and hippocampus.

Streptozotocin model: One-factor ANOVA analysis showed that $A\beta_{1-42}$ content in frontal cortex ($F_{2,20}= 16.2$, $p<0.001$, $\eta p2= 0.42$) and hippocampus was different, compared to the experimental group ($F_{2,20}= 15.9$, $p<0.001$, $\eta p2= 0.575$), in tissues isolated from the first experimental model. Statistical processing of the data showed that the STZ-veh group was characterized by increased amounts of $A\beta_{1-42}$ in frontal cortex and hippocampus compared to the sham-veh group ($p= 0.05$). Ago treatment restored the balance of $A\beta_{1-42}$ back to control values in both brain structures, which was established by the results reported in the STZ-Ago group ($p<0.001$) (Figure 14).

Amyloid model: a significant difference between the groups in terms of $A\beta_{1-42}$ level was demonstrated, both in FC (Kruskal-Wallis test: $H= 13.524$, $p= 0.004$) and hippocampus in animals of the second experimental model (one-factor ANOVA analysis: $F_{3, 28}= 10.259$, $p< 0.001$). In hippocampi isolated from the Aβ-veh group we observed a significant increase in the amount of $A\beta_{1-42}$ compared to the sham-veh group ($p= 0.0004$) (Figure 15 B). Statistical analysis confirmed that both treatment approaches (experiments 1 and 2) were successful in preventing the accumulation of $A\beta_{1-42}$ in the brain stuctures we examinated.

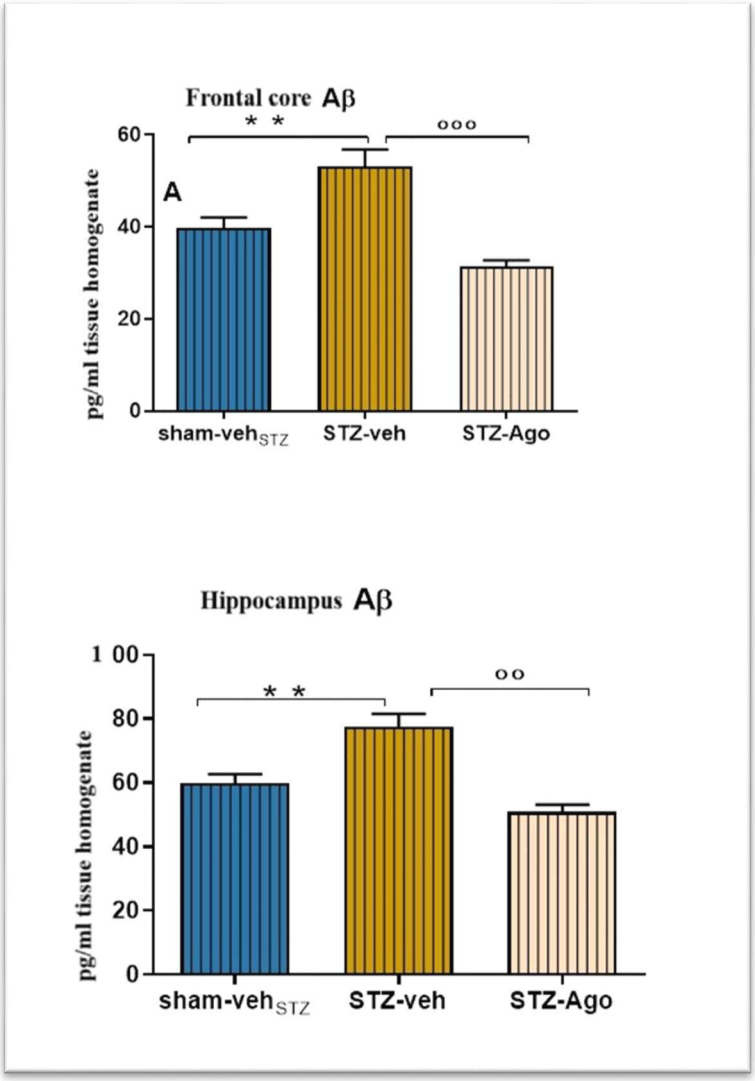


*Figure 12. Effect of Ago on $A\beta_{1-42}$ levels, presented in pg/ml tissue homogenate, in FC and hippocampus. *comparison of STZ-veh with sham-veh; comparison of STZ-Ago with STZ-veh.*

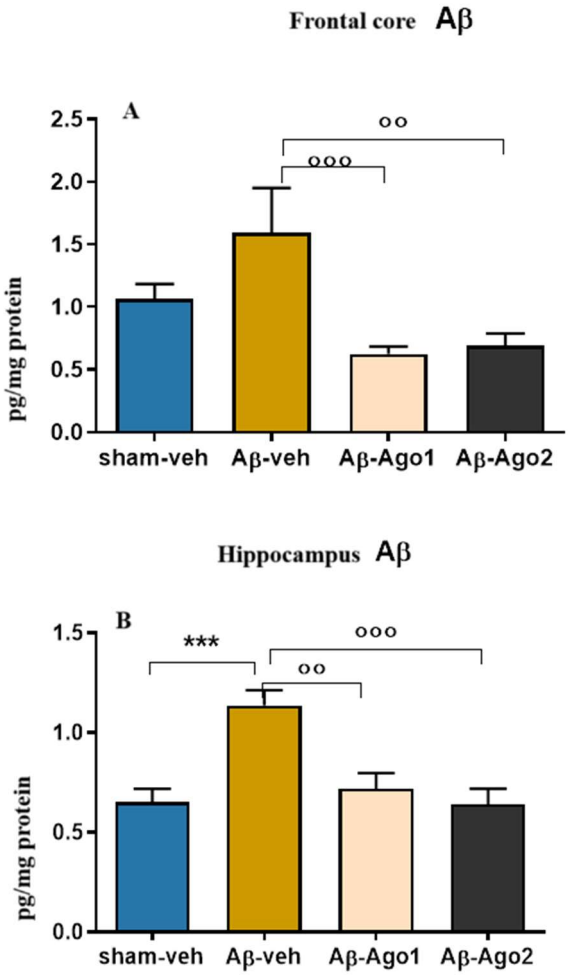


*Figure 13. Effect of Ago on $A\beta_{1-42}$ levels, presented in pg/mg protein, in FC and hippocampus. *comparison of Aβ-veh with sham-veh;° comparison of Aβ-Ago1 and 2 with Aβ-veh.*

2.2 Effects of agomelatine on tau-protein levels in FC and hippocampus (Streptozotocin model).

We determined tau protein content in brain tissue by Western blot. The AD group showed significantly increased levels of the marker compared with the control, sham-operated group (***p< 0.001). The Ago-treated group reduced tau levels sufficiently, in the FC and hippocampus compared to the STZ-veh group ($p<0.001$; $p<0.01$)(Figure 16). The effect of STZ was equally noticeable in both structures studied, and Ago treatment reduced the deposition of hyperphosphorylated tau protein slightly more markedly in the FC.

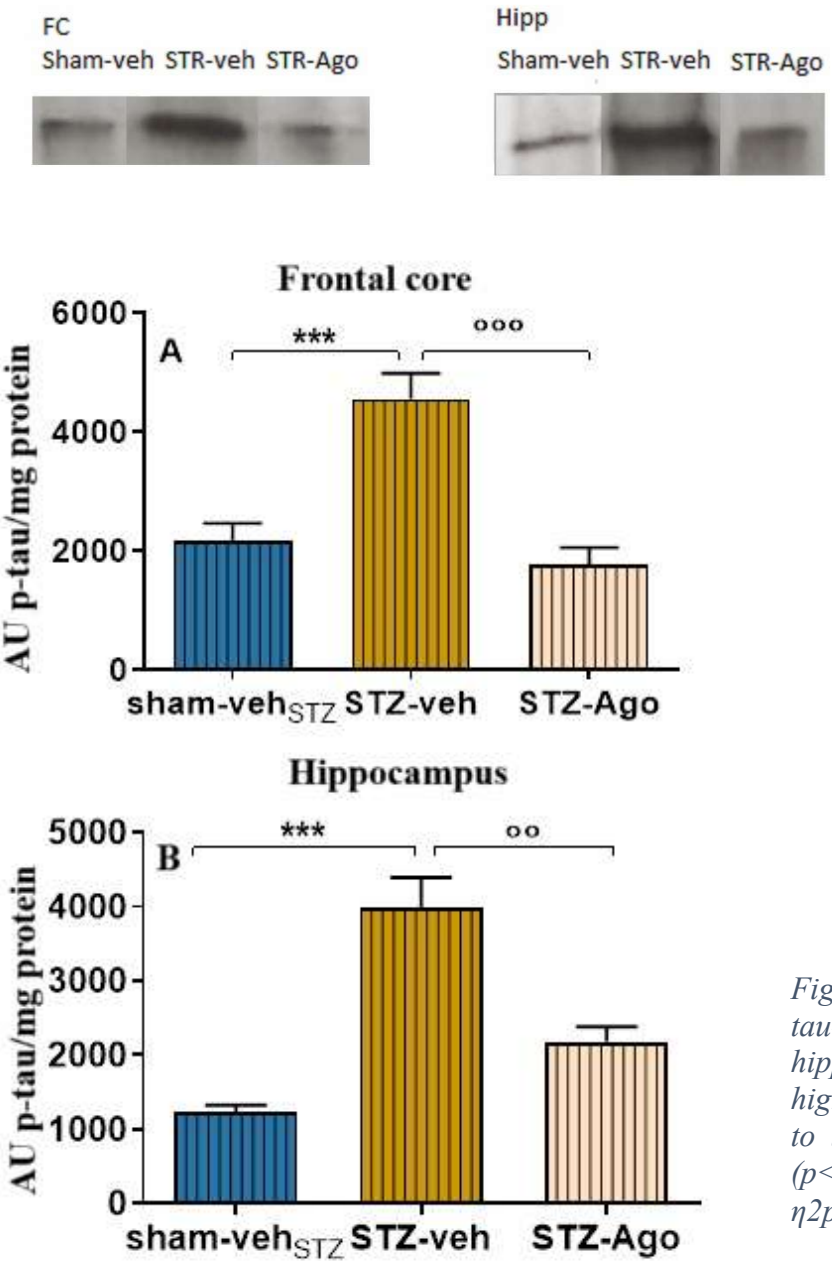


Figure 14. Effect of Ago treatment on tau-protein content in FC and hippocampus. Levels were significantly higher in the STZ-veh group compared to the sham-veh and STZ-Ago group ($p<0.001$, $\eta 2p=0.68$ and $p<0.001$, $\eta 2p=0.75$).

2.3. Effects of agomelatine on signaling markers of inflammation: TNF-α and IL-1β, in FC and hippocampus (STZ model).

Relative to TNF-α levels, the Kruskal-Wallis test demonstrated a significant difference between groups in both frontal cortex (H= 7.5, p= 0.023) and hippocampal samples (H= 13.6, p= 0.001). The STZ-veh group had an increased TNF-α level in both of these structures.

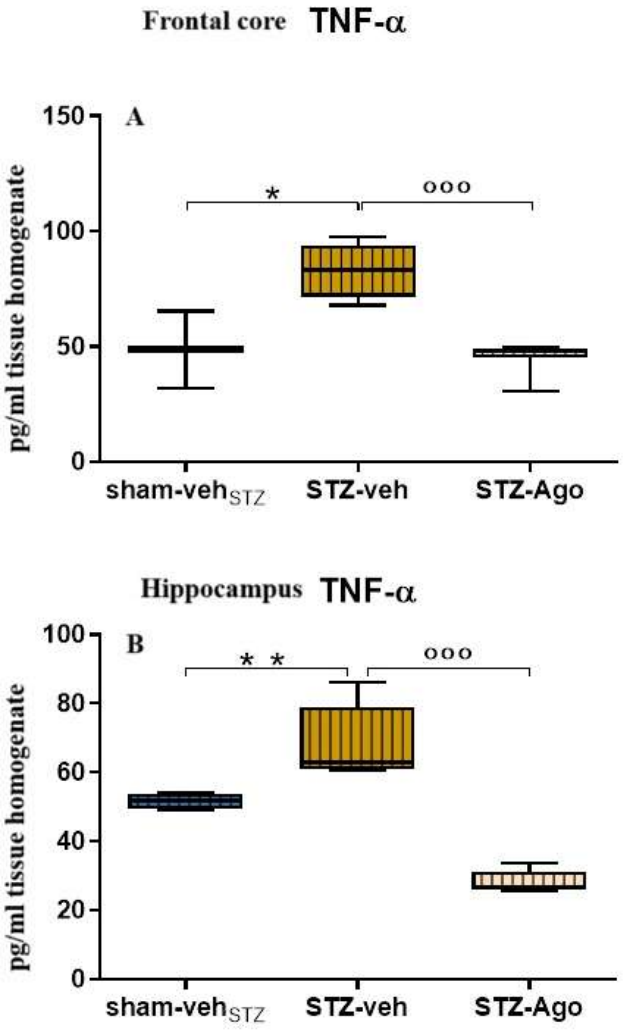


*Figure 15. Measured TNF-α levels in the FC and hippocampus. STZ-veh group showed increased levels of the proinflammatory marker in both brain structures, relative to both controls and Ago-treated animals (**p<0.01;***p<0.001); *comparison of STZ-veh with sham-veh; comparison of STZ-Ago with STZ-veh.*

Measured levels of the proinflammatory cytokine IL-1β, were also significantly higher in the STZ-veh group compared to the control sham-vehSTZ , again in both frontal cortex (p= 0.02) and hippocampus (p< 0.001). The STZ-Ago group was characterized by TNF-α and IL-1β values lower than those reported in the sham-vehSTZ group, in brain homogenates from both areas studied (p<0.001) (Figures 17 and 18).

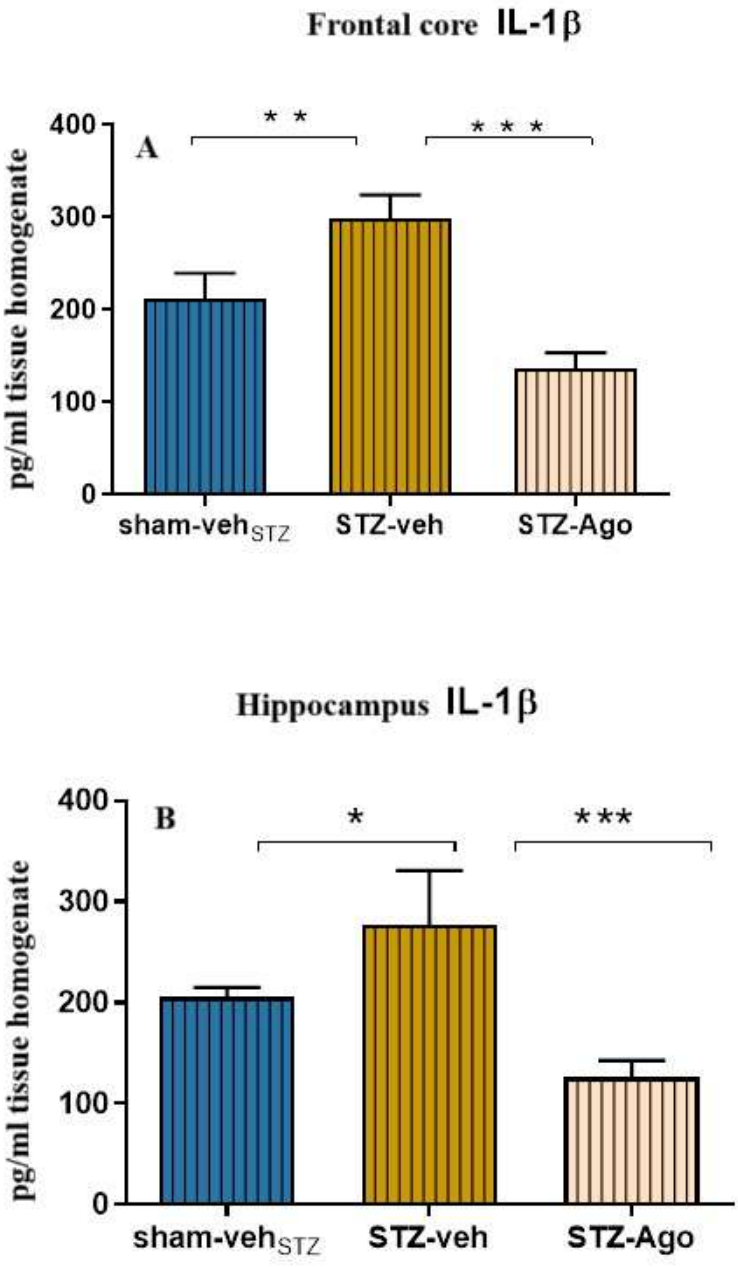


*Figure 16. Measured IL-1β levels in the FC and hippocampus. STZ-veh group showed increased levels of the proinflammatory marker in both brain structures compared to controls and Ago-treated animals (**p<0.01;***p<0.001);*comparison of Aβ-veh with sham-veh;°comparison of Aβ-Ago with Aβ-veh.*

2.4. Secretase enzymes (Amyloid model)

2.4.1. Alpha secretase

The concentration of α-secretase was not affected by icv$A\beta_{1\text{-}42}$ infusion in either the FC (one-factor ANOVA: $F_{3,28}$= 0.116, p= 0.95) or the hippocampus (Kruskal-Wallis test: H= 6.07, p= 0.108) (Figure 19 A, B). However, AβAgo1 induced a significant increasein α-secretase levels in the hippocampus compared to the sham-veh group (p= 0.0213) and the Aβ-veh group (p= 0.0324).

2.4.2 Beta-secretase

Infusion of icv$A\beta_{1\text{-}42}$ did not affect β-secretase concentration in either the FC (Kruskal-Wallis test: H= 3.477, p= 0.324) or hippocampus (one-factor ANOVA: F3,28= 0.829, p< 0.505)(Figure 19 C, D)

2.4.3 Gamma secretase

The reported difference between groups in terms of γ-secretase concentration in FC was not significant (Kruskal-Wallis test: H= 1.919, p= 0.589). However, the concentration of the same enzyme in the hippocampus was more significantly affected after intracerebroventricular infusion of $A\beta_{1\text{-}42}$ (one-factor ANOVA: $F_{3,28}$= 4.469, p= 0.013). The amount of γ-secretase was significantly increased in the Aβ-veh group compared to the sham-veh group (p= 0.032), and in the Aβ-Ago1 and Aβ-Ago2 groups, it fell to that of controls (p= 0.041 Aβ-Ago1 compared to the Aβ-veh group) and (p= 0.0024 Aβ-Ago2 compared to the Aβ-veh group).

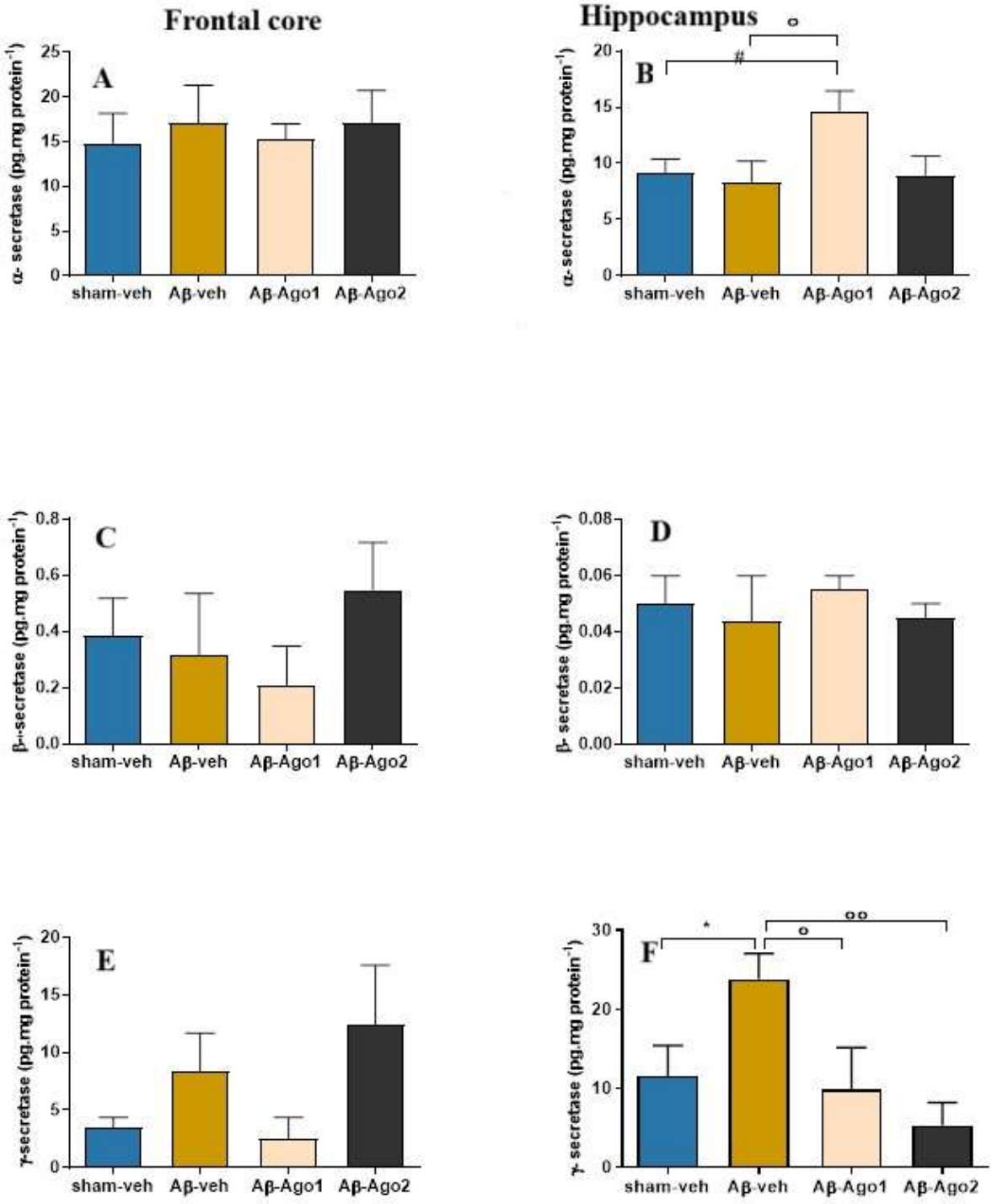


*Figure 17. Effect of chronic Ago treatment on α-secretase concentration (pg/mg tissue) in FC (A) and hippocampus (B), on β-secretase concentration (pg/mg tissue) in FC (C) and hippocampus (D), on γ-secretase concentration (pg/mg tissue) in FC (E) and hippocampus (F). *P= 0.0213 Aβ-Ago1 compared with sham-veh group, *P= 0.0324 Aβ-Ago1 compared with Aβ-veh group (D);*P= 0.032 Aβ-veh group compared with sham-veh group, *P= 0.041 Aβ-Ago1 compared with Aβ-veh group, **P= 0.0024 Aβ-Ago 2 compared with Aβ-veh group (E, F). *comparison of Aβ-veh with sham-veh;° comparison of and 2 with Aβ-veh; # comparison between Aβ-Ago1 and sham-veh.*

3. Effect of agomelatine treatment on neuronal loss.

4.1. Morphological effects of agomelatine on different brain structures in STZ model of AD

4.1.1. Changes observed in neuronal populations in the dorsal hippocampus

The extent of cell damage in the dorsal hippocampus is clearly visible in the attached figures (Figure 20). The average number of cells per unit area does not differ between the STZ-veh and STZ-Ago groups in the CA1 (septal, septo-temporal, and temporal), CA2 temporal, CA3a and CA3c (septal, septo-temporal, and temporal), CA3b (septal and septo-temporal), and *girus dentatus* hilus (septal, septo-temporal, and temporal) fields of the dorsal hippocampus.

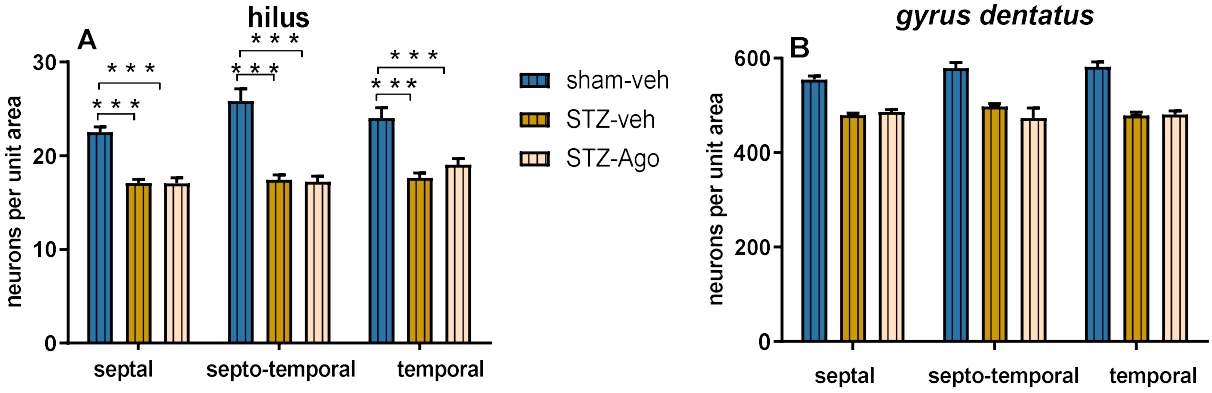


*Figure 18. The mean number of cells per unit area showed no statistically significant differences between the STZ-veh and STZ-Ago groups in the hilus of the girus dentatus (septal, septo-temporal and temporal) of the dorsal hippocampus, but in the hilus (septal and septo-temporal), the effect of Aβ (septal,septo-temporal and temporal areas **p<0.01***p<0.001) was reported.*

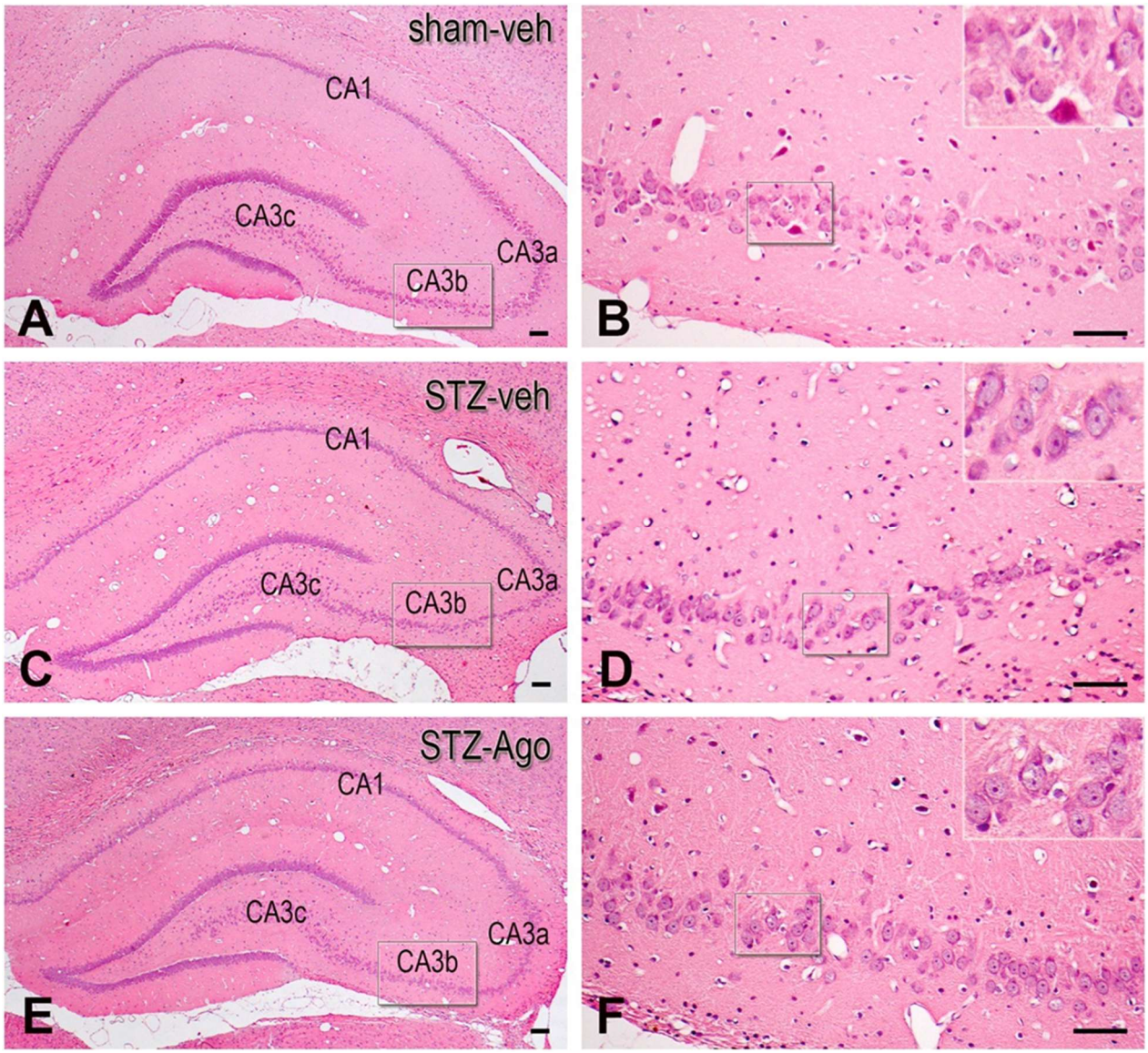


Figure 19. Morphological effects of Ago on the hippocampal neuronal population.(A) Overview of hippocampus in sham-operated and vehicle-treated rats showing intensely H&E-stained pyramidal neurons in all hippocampal subfields.(B) Increased CA3b field. The insets show the morphology of the neuronal population in the corresponding hippocampal area shown in the small rectangles. (C) Microscopic image of an H&E-stained section of the dorsal hippocampus in STZ rats treated with vehicle. (D) Hippocampal CA3b field at higher magnification (insets). (E) Hippocampal formation in STZ rats treated with Ago. Details of the enclosed areas in (F) reveal partial recovery of pyramidal neurons in the CA3b subfield of the hippocampus. Scale bars=200 μm in A, C, E; 50 μm in B, D, F in higher magnification insets.

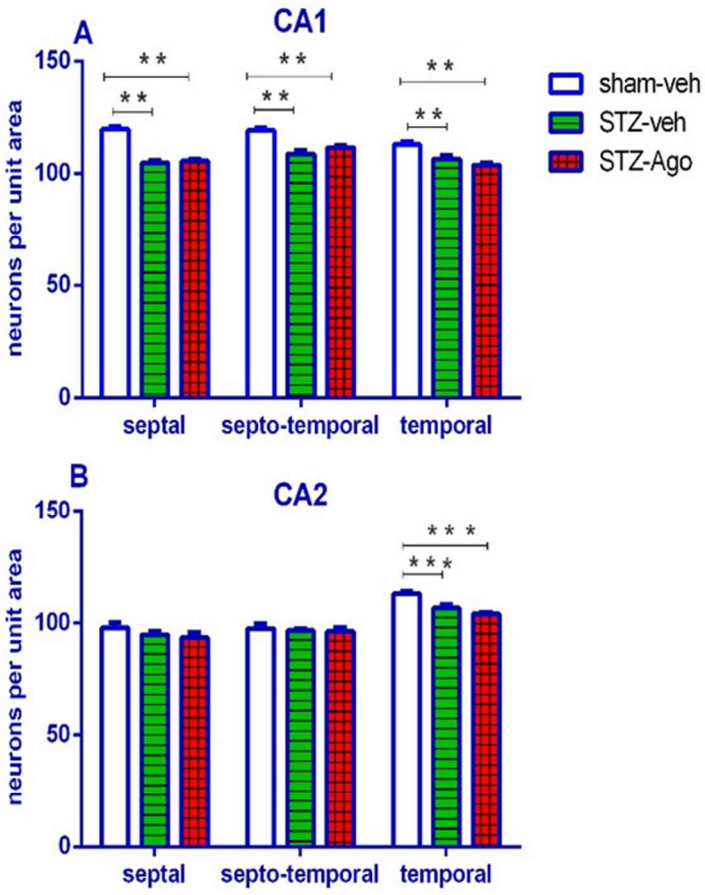


*Figure 20. Mean number of cells per unit area did not differ between STZ-veh and STZ-Ago groups in CA1 septal, septo-temporal, and temporal (septal and septo-temporal area **p<0.01), temporal CA2 (***p<0.001) fields.*

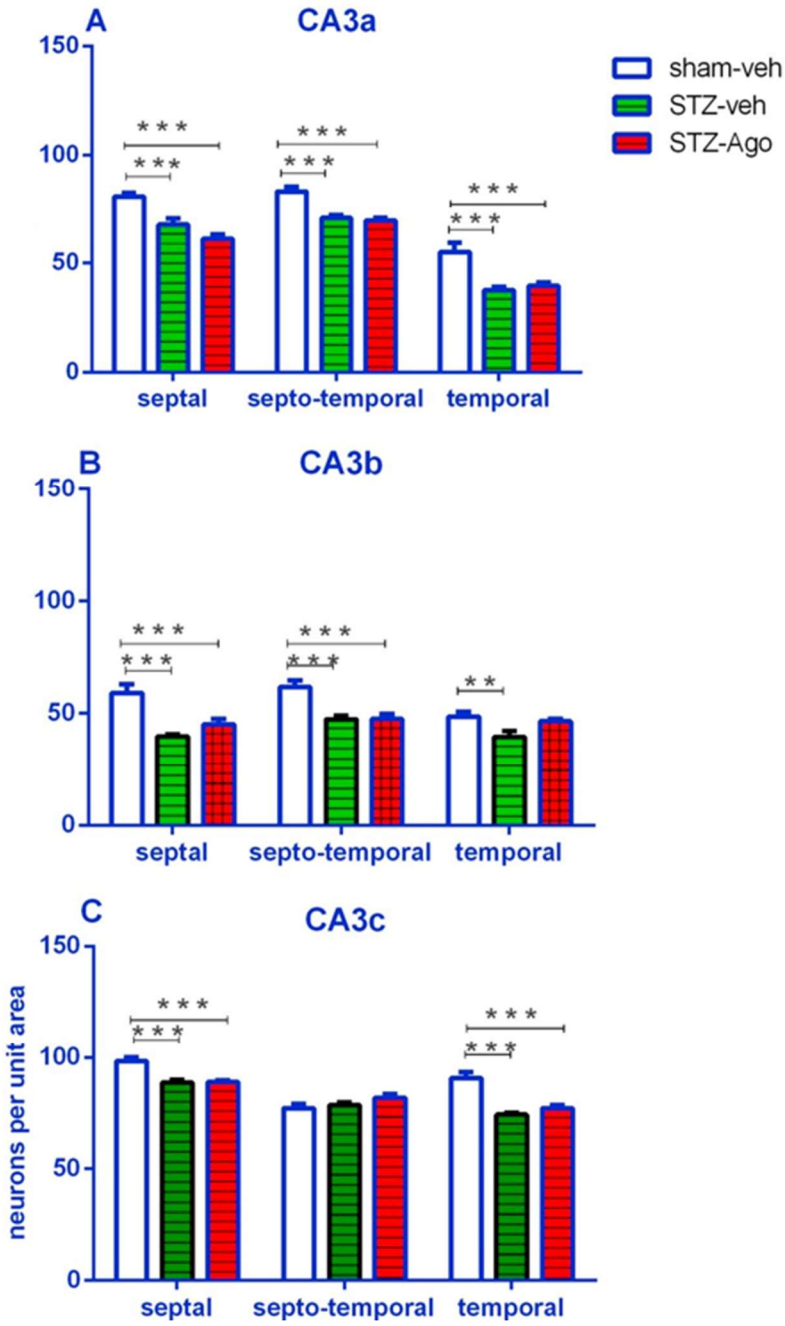


Figure 21. The mean number of cells per unit area did not differ between the STZ-veh and STZ-Ago groups in CA3a and CA3c (septal, septo-temporal, and temporal), CA3b (septal and septo-temporal).

4.1.2. Changes observed in neuronal populations in the basolateral nucleus of the amygdala and piriform cortex

The rate of neuron loss per unit area in the septal and septo-temporal areas in the basolateral nucleus of the amygdala (BL) and pyriform cortex (Pir) was similar in the STZ-veh and STZ-Ago groups (Fig. 24 A, B). Chronic treatment with Ago had a neuroprotective effect, most prominent in the hippocampal CA3b temporal field (Fig. 23B) and septal piriform cortex (Fig. 22 B).

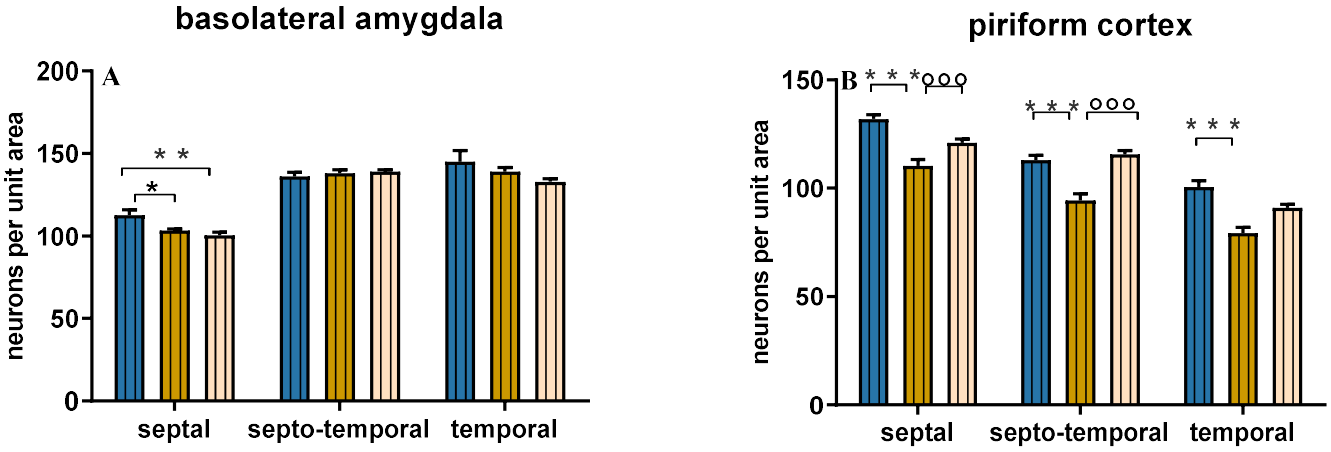


*Figure 22. Effects of Ago treatment, in BL and Pir: the number of neurons per unit area was not altered in the septo-temporal and temporal parts of BL, and in the septal Ago failed to restore their number. The toxic effect of Aβ was clearly evident in all three areas of Pir examined, which in the Aβ-Ago groups was significantly alleviated by the action of Aβ (septal and septo-temporal area ***p<0.001).*

4.2 Morphological effects of agomelatine on different brain structures in an amyloid model of AD

Cell density in the hippocampus is shown in Figure 25 (A-O). A significant difference between the three groups was found for cell density in the dorsal and ventral hippocampus (one-factor ANOVA: $p<0.05$). Intracerebroventricular infusion of $A\beta_{1-42}$ induced significant cell loss in a large proportion of dorsal hippocampal subfields (septal, septo-temporal and temporal). In CA1, CA2, CA3a, and septo-temporal and temporal CA3b, as well as in CA3c, and the CA1 (vCA1) and vCA2 subfields of the ventral hippocampus, the number of neurons per unit area was also significantly reduced ($p=0.047$ and $p<0.001$, respectively, compared with the sham- veh group).Figure 25 (A-F)

Agomelatine treatment initiated simultaneously with icv infusion of $A\beta_{1-42}$ exerted a strong neuroprotective effect in the septal and temporal CA1 subfield ($p<0.001$, compared to the Aβ-veh group), while this melatonin analog tended to ameliorate Aβ1-42-induced cell loss in the septal CA1 and temporal CA3c subfields of the hippocampus ($p=0.006$ compared to the sham-veh group and $p<0.001$ compared to the Aβ-veh group, respectively) (Figure 25, 26 A-F). However, the drug was unable to ameliorate icv$A\beta_{1-42}$-induced neuronal losses in the CA2, CA3a, septo-temporal and temporal CA3b subfields of the dorsal hippocampus and vCA2 subfield ($p>0.05$ compared to the sham-veh group). Furthermore, while the mean number of cells per unit area did not differ between the three groups in the *girus dentatus*, septal and temporal *hilus* (one-factor ANOVA: $p>0.05$), a significant difference was found for the septo-temporal *hilus* ($F_{2,43}= 4.292$, $p=0.020$) (Figure 26G,H). Post hoc analysis showed reduced cell density in the *hilus* of the Aβ-veh group ($P= 0.016$ compared with the

sham-veh group) (Figure 25 D, I). Ago treatment did not affect Aβ1-42-induced cell damage in this area.

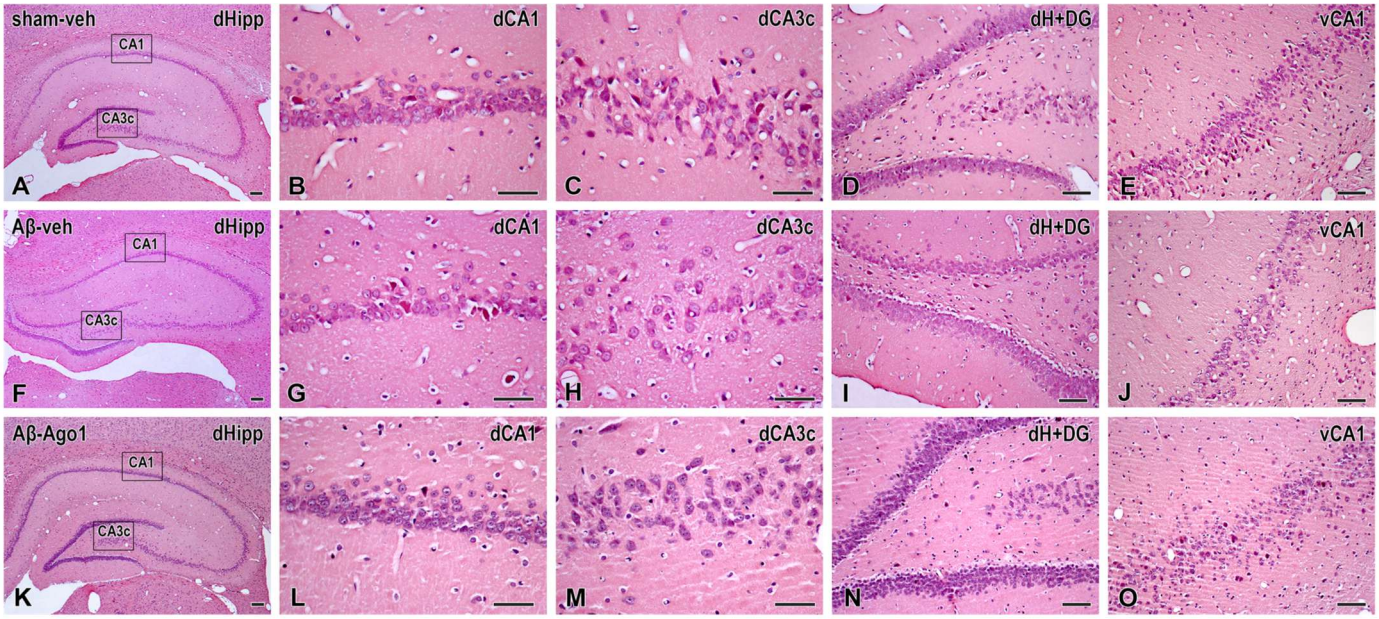


Figure 23. Representative hematoxylin and eosin (H&E)-stained coronal sections of the dorsal (dHipp) and ventral (vHipp) hippocampal formation in (A-E) rats: sham-operated, vehicle-treated (sham-veh), (F-J) Aβ1-42-treated, vehicle-treated (Aβ-veh) and (K-O) Ago-treated (Aβ-Ago1) rats. Representative micrographs in the second and third columns are higher magnifications of the enclosed regions of the images in the first column than the CA1 and CA3c regions of dHipp, respectively. The images in the fourth column visualize the hilus of DG with densely packed granule cells. The microscopic images in the last fifth column illustrate pyramidal neurons of the ventral hippocampal CA1 subfield. Aβ-veh rats (F-J) show loss of neurons in the pyramidal cell layers dCA1 (G), dCA3 (H), and vCA1 (J) compared with control sham-veh rats. (I) Granule cells in dHIPP are also damaged. (K). Small increase in dorsal hippocampus in Aβ-Ago1 rats. Details of boxed areas in (K) reveal partial recovery of pyramidal neurons in the dCA1 (L), dCA3c (M) and vCA1 (O) subfields and the dHipp (N) hilus. Scale bar = 50 μm.

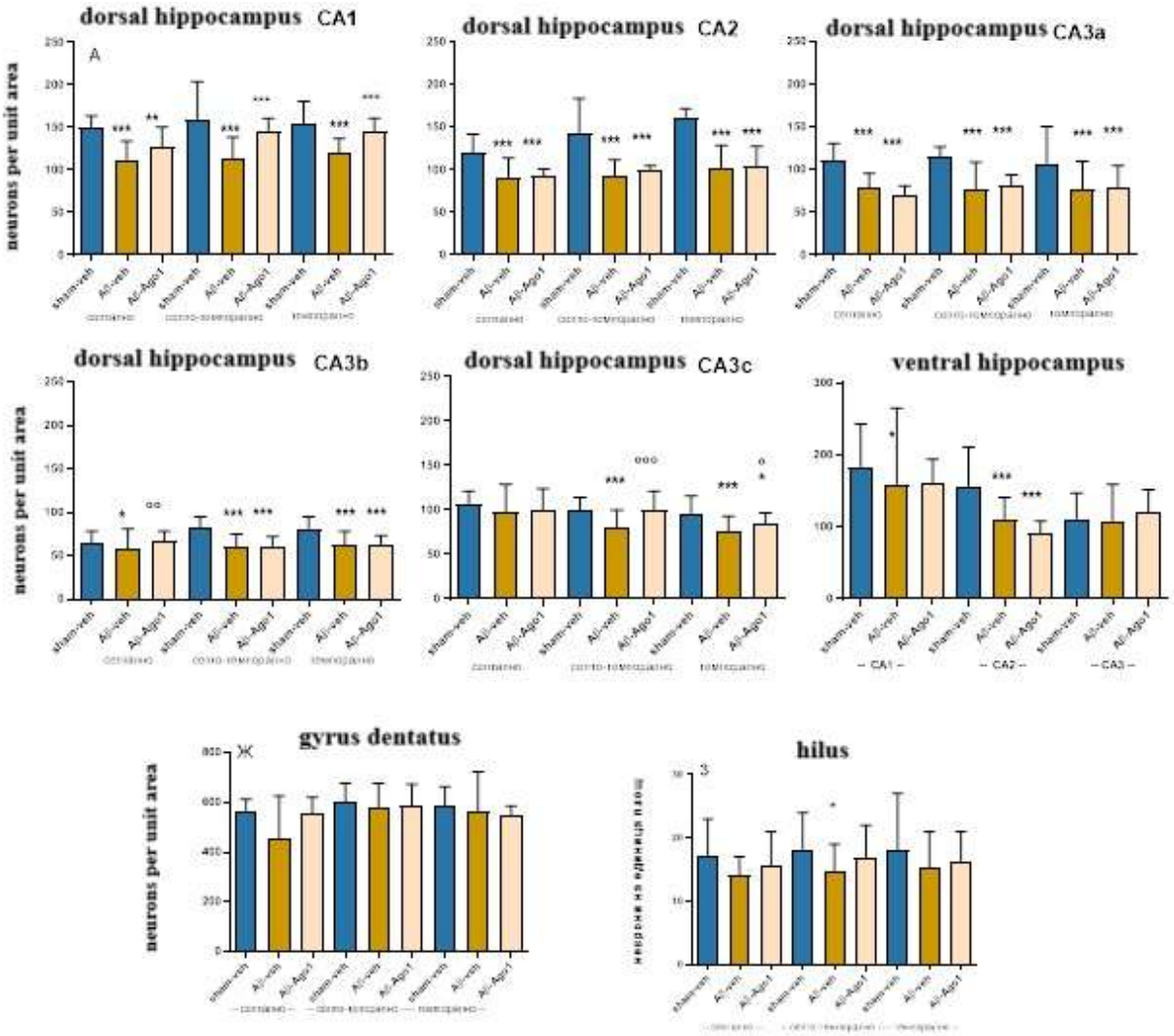


*Figure 24. Effects of chronic Ago treatment on septal, septo-temporal and ventral subfields of CA1, CA2, CA3a, CA3b, CA3c of the dorsal hippocampus (A-D) and subfields of CA1, CA2 and CA3 of the ventral hippocampus (vCA1, vCA2 and vCA3) (F), septal, septo-temporal and ventral DG and hilus of DG (G, H). Data are presented as means±S.E.M. ***p<0.001 Aβ-veh compared with sham-veh group; *p= 0.047 Aβ-veh compared with sham-veh group; *p= 0.03; Aβ-Ago1 compared with sham-veh group; **p= 0.006; Aβ-Ago1 compared with sham-veh group; ***p< 0.00;1 Aβ-Ago1 compared with the sham-veh group; ***p= 0.03 Aβ-Ago1 compared with the Aβ-veh group; ***p< 0.001 Aβ- Ago1 compared with the Aβ-veh group; *p= 0.016 Aβ-veh compared with the sham-veh group. (Kruskal-Wallis H test).*

Cell densities in BL and Pir are shown in Figures 27 and 28. The Kruskal-Wallis test showed a significant difference in cell density per unit area between the three groups for the septal, septo-temporal, and temporal regions of BL and Pir (H= 34.059 and H= 34.091, respectively). Mann-Whitney U test showed that icv infusion of $A\beta_{1-42}$ induced significant cell loss in these brain regions ($p<0.001$). The damage was not corrected by Ago treatment in BL, whereas this melatonin analogue induced partial neuroprotection in the septal (p= 0,037 Aβ-Ago1 compared with Aβ-veh and $p<0.001$ Aβ- Ago1 compared with the sham-veh group) and temporal Pir (p= 0.011 Aβ- Ago1 compared with Aβ-veh and $p<0.01$ Aβ-Ago1 compared with the sham-veh group) (Fig. 27 A-F).

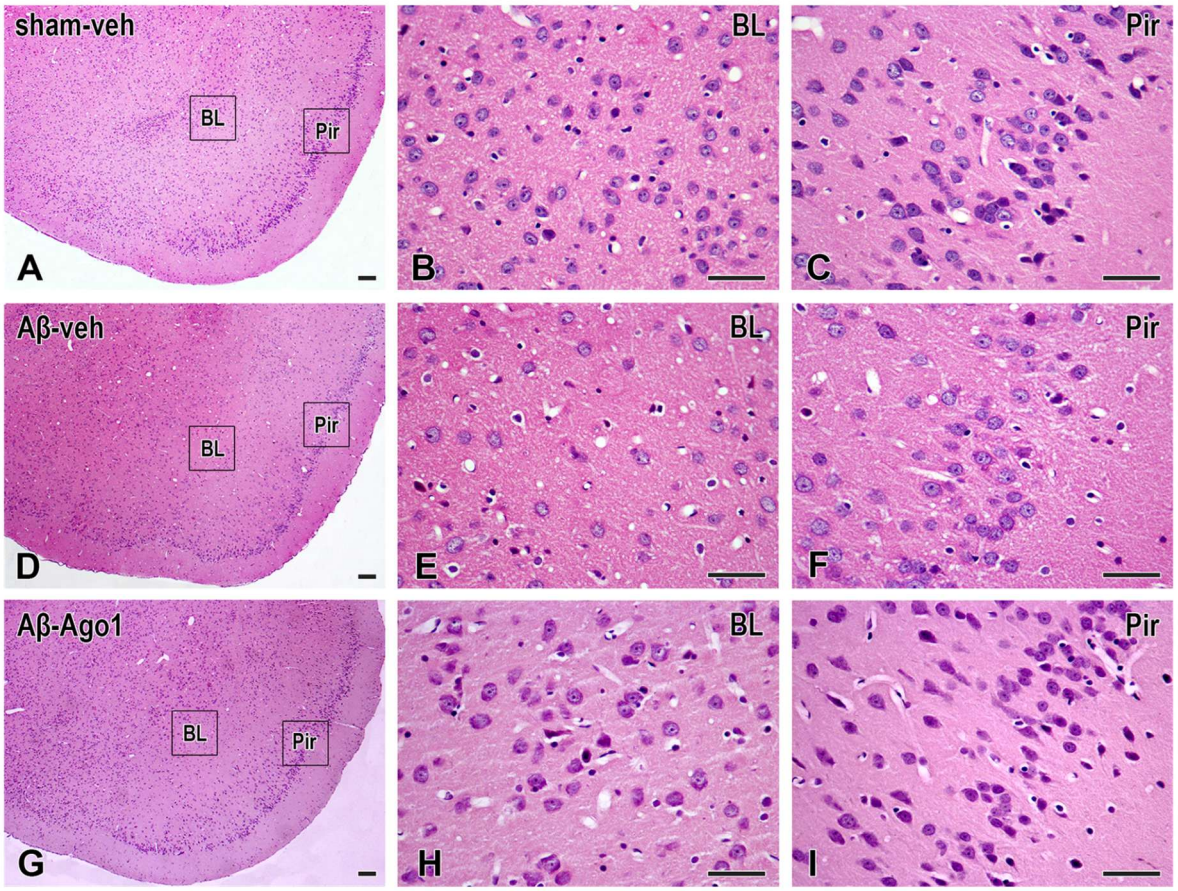


Figure 25. Morphological effects of agomelatine on pyriform cortex (Pir) and basolateral amygdala (BL) in rats. (A) Representative H&E-stained sections at the level of BL and Pir in vehicle-treated control rats (sham-veh). Higher magnification of the boxed areas of the image in (A) showing the normal morphology and neuronal population density in BL (B) and Pir (C) in sham-veh-treated rats. Small (D)- and large (E, F) increases in BL and Pir in Aβ1-42 vehicle-treated (Aβ-veh) rats. Apparent loss of neurons in BL (E) and Pir (F) compared to the sham-veh control group. (A) View with small increase in BL and Pir in the Aβ-Ago1 group. (G) Larger magnification of the boxed areas in the image in (G) shows the protective effect of Ago on neuronal survival in Pir (I), whereas in the basolateral nucleus of the amygdala (H) the effect is not as strong. Scale bar = 50 μm.

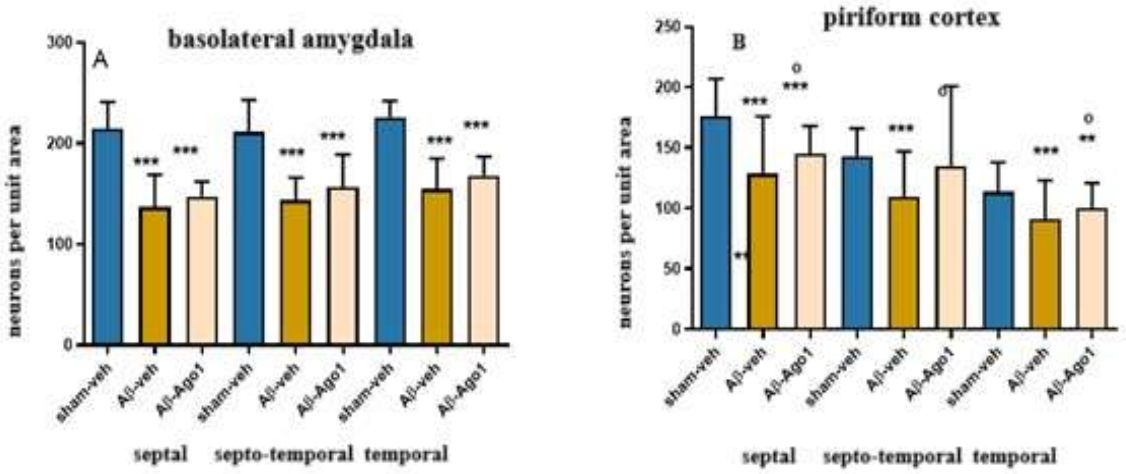


*Figure 26. .Effect of chronic Ago treatment on septal, septo-temporal and ventral BL and pyriform cortex. Data are presented as $\bar{x}$ ± S.E.M. ***p<0.001 Aβ-veh and Aβ-Ago1 compared with sham-veh group; **p<0.01 Aβ-Ago1 compared with sham-veh group; °p= 0.011 Aβ-Ago1 compared with Aβ-veh group in Pir. (Kruskal-Wallis H test).*

Discussion

The present study revealed that chronic Ago treatment positively influenced the pathogenesis associated with cognitive impairment and increased anxiety and restlessness, exerting neuroprotective and anti-inflammatory effects, in two rat models of sAD induced by icv injection of STZ or Aβ.

Experimental models that can accurately mimic the pathology of sAD development in humans are important for testing new therapeutic approaches to sAD treatment. Commonly used transgenic mouse models of AD provide valuable insights into the molecular mechanisms underlying cognitive impairment, but due to manipulation of genes associated with the β-amyloid protein, they resemble the familial but not the sporadic form of AD (Salkovic-Petrisic et al., 2013). Due to the fact that a small percentage of all AD cases are genetically inherited, transgenic models are not particularly suitable for the study of sAD. In our studies, we selected two of the most commonly used animal models for sAD induction, generated by icv injection of STZ or Aβ, due to the fact that they have been extensively studied and validated by a number of teams, to partially balance the limitations that each has, and due to the heterozygous nature of AD, with the idea of testing whether Ago would affect similar symptomatology predicted by different factors.

Three months after the introduction of STZ and in the early stages after the introduction of Aβ1-42 into the brain ventricles, we found significant behavioral changes, such as anxiety, anhedonia, and cognitive impairment, increased levels of Aβ, phosphorylated tau protein, and proinflammatory signaling molecules (TNF-α and IL-1β) in the frontal cortex and hippocampus, as well as neuronal loss in the dorsal hippocampus, BL, and Pir in the solvent-treated control group.

The rat icv STZ model is an experimentally validated model that mimics the behavioral and biochemical changes of sAD by inducing impaired insulin signaling pathways in the brain and reduced glucose metabolism in the cortex and hippocampus (Hoyer & Lannert, 2008; Talebi et al., 2025). This leads to cholinergic deficits and cognitive dysfunction (Salkovic-Petrisic et al., 2014). Knezovic et al. (2015) reported that the icv model of STZ in rats is characterized by time- and dose-dependent deficits in cognitive functions and concomitant plaque formation from Aβ and intracellular deposits of hyperphosphorylated tau protein. The results of this dissertation confirmed previous findings (Firoozi et al., 2023; Hoyer & Lannert, 2008; Shingo et al., 2013) that three months after icv STZ infusion at a high dose of 3 μg/structure is a critical time interval for detection and accumulation of Aβ in the frontal cortex and hippocampus.

Regarding the amyloid model, the cytotoxicity of Aβ1-42 upon icv injection is related to its ability to self-aggregate into fibrillar oligomers and plaques (Vadukul et al., 2017). The pathological effect we reported is consistent with reports of other teams (Sharma et al., 2016) administering it in a protofibrillar form. There is evidence that the introduction of Aβ1-42 into the brain negatively affects learning ability and leads to the degeneration of cholinergic neurons, making Aβ-treated rats a suitable animal model for sAD (Nitta et al., 1994). The etiology remains unclear, but STZ-injected icv induces sAD-like pathology by altering glucose metabolism, leading to impaired insulin signaling, synaptic dysfunction, tau protein hyperphosphorylation, Aβ deposition, and neuronal apoptosis. A similar pathological effect is observed with icv injection of Aβ (Puzzo et al., 2014). Thus, STZ and Aβ-induced experimental models are well suited for investigating the underlying molecular and pathophysiological mechanisms of sAD and testing novel therapeutic approaches (Shingo et al., 2013). In both models, we performed the behavioral assays as early as 16 h after agomelatine administration to avoid its acute effect and to base our conclusions on its induced neuronal plasticity.

Anxiety and depression are comorbid psychological symptoms of AD, affecting around 90% of patients (Bingley et al., 2025). Most experimental studies have focused on behavioural changes associated with cognitive function, and only a few reports have addressed changes associated with anxiety and depression. It has been reported that transgenic mice (3xTg-AD), a nontransgenic model (OXYS) of sAD (Rudnitskaya et al., 2015) as well as icv STZ/Aβ mice and Wistar rats have increased anxiety-like symptoms as reported by EPM and/or OF tests (Y. Chen et al. 2013; Gutierres et al. 2014; Pinton et al. 2011; Sterniczuk et al. 2010). In the present study, we showed that Ago treatment attenuated the increased anxiety behavior in rats from both sAD models. Recently, Rudnitskaya et al. (2015) reported that melatonin administration ameliorated changes in anxiety level of OXYS rats tested by EPM by increasing the number of entries and time spent in open spaces. Fear responses were associated with amygdala hyperactivity. It is part of the limbic system and is involved in threat recognition, emotional memory, and the regulation of stress responses (Forster et al., 2012). Ago is unable to restore the cell loss induced by STZ and Aβ1-42, suggesting a lack of association between the anxiolytic effect of this melatonin analogue and neuronal damage in the amygdala.

The anxiolytic and antidepressant effects of this atypical antidepressant have been established in animal models of neurodegenerative disorders as well as in humans (Kennedy, 2007; Martinotti et al., 2016; Ownby et al., 2006; Tchekalarova et al., 2016, 2017). One hypothesis for the underlying mechanism of AD comorbid depression suggests the role of altered Aβ metabolism (Mahgoub & Alexopoulos, 2016; Namekawa et al., 2013). In this regard, Ishijima and colleagues (Ishijima et al., 2018) reported a positive association between elevated plasma cortisol and an imbalance in Aβ metabolism in patients with severe depression. In our 2016 study, we reported that the same Ago treatment protocol exerted an antidepressant effect and restored the impaired feedback mechanism of corticosterone secretion in a dexamethasone suppression test of pinealectomized rats (Tchekalarova et al., 2016). The hyperactivated HPA axis is considered an important marker of severe depression. A limitation of the present study is that the effect of Ago treatment on altered corticosterone levels in normal and following stress, which reflects the degree of HPA axis activation, was not analyzed to test the hypothesis of a positive relationship between altered Aβ metabolism and impaired hormone secretion. This issue deserves future investigation.

The brain is characterized by an extremely intense metabolism. Although it accounts for only about 2% of body weight, it consumes approximately 20% of the body's total resting energy. It is therefore highly dependent on oxygen and glucose concentrations, but its antioxidant capacity is relatively low, making it vulnerable to a variety of internal and external factors that can alter its metabolite balance (Lei et al., 2024). The incidence and predisposition to AD-specific pathogenesis is seen in aging and susceptibility to inflammatory processes in the brain, pathologically activated microglia and astrocytes, and disruptions in BBB integrity due to damage to the brain endothelium (W. Cai et al., 2017). We found that chronic Ago treatment reduced Aβ accumulation as well as levels of proinflammatory signaling molecules, TNFα and IL-1β in the frontal cortex and hippocampus of rats in the streptozotocin model. To our knowledge, this is the first study to report the effect of this melatonin analogue, used clinically as an antidepressant, on neuropathological sequelae resembling AD symptoms (Yang et al., 2021).

The anti-inflammatory properties of melanin, whose structural analogue is agomelatine, have been repeatedly demonstrated in the scientific literature (Cardinali et al., 2002; Roy et al., 2022). STZ has been shown to induce neuronal death and increase levels of proinflammatory cytokines (IL-6 and TNF-α) (Gerzson et al., 2020). Neuroinflammation is considered a crucial stage in the development of

the neurodegenerative process of AD (Buckwalter and Wyss-Coray, 2004). In the streptozotocin model, it is detected one week after low dose STZ injection (Nazem et al., 2015; Solmaz et al., 2015) reported that TNF-α increased in rat brain two weeks after icv STZ (Sabbagh et al., 2013). We demonstrated that TNF-α and IL-β are increased three months after icv STZ and that chronic Ago treatment suppresses these inflammatory signaling molecules. Recently, we found that Ago treatment elicits a strong anti-inflammatory response in both the periphery (plasma IL-1β) and the brain (attenuation of gliosis) in an experimental model of epileptogenesis (Tchekalarova et al., 2018). In summary, when examining the effect of Ago on the pathology of the streptozotocin model, we found improved spatial memory, reduced TNF-α and IL-1β levels, reduced neuronal loss, and suppression of Aβ accumulation in the hippocampus.

In the amyloid model, the anxiolytic effect of Ago was demonstrated by correcting the induced reduction in time spent in aversive areas in both the OF and EPM tests, as well as by reducing the number of transitions in the LDT test. The antidepressant action of Ago was confirmed in the SPT and FST with a reduction in pattern-specific anhedonia and a behavioral response of "despair."Depressive-like behavior in the Aβ-veh group was also associated with reduced locomotor activity, which was shown in OF (total distance) and EPM (number of entries into closed and open arms), and this abnormal behavioral response was also mitigated by Ago. Although the increased immobility time of the Aβ-veh group in the FST may be related to their reduced motor activity found in two of the other behavioural tests, the OF and EPM, the lack of preference for sweet solutions leads us to judge that this is an authentic depressive response. These results are consistent with those obtained in the icvSTZ model of sAD and further evidence that the Ago treatment protocol applied early in the progression of the pathology may alleviate anxiety (stay and number of open arm entries) in the EPM and depressive-like behavior (reduced motor activity and anhedonia in the sweet solution preference test). Results showed that Ago exerted beneficial effects on concomitant behavioral impairments and elicited domain-specific neuroprotection. Both treatment approaches (administration of Ago in the early or late stage of AD) successfully attenuated icvAβ1-42-induced accumulation of this toxic peptide in the FC and hippocampus mainly by reducing γ-secretase concentration. Extracellular Aβ peptide accumulation is a prerequisite for cellular damage in selected structures associated with various behavioral functions, including affective responses and cognitive abilities. We found cell loss in certain brain structures in the Aβ-veh group associated with anxiety and depression, such as the ventral hippocampus and BL. Surprisingly, Ago treatment failed to prevent these morphological changes, suggesting that the anxiolytic and antidepressant effects exhibited are mediated through other mechanisms. Our results confirm the observations of previous studies showing cell loss in the hippocampus, hilus, BL, and Pir as a consequence of icv treatment with Aβ1-42 (He et al., 2013). Similar to the results obtained with STZ, the Ago amyloid model showed weak and structure-specific neuroprotection. While in the streptozotocin model, Ago treatment positively affected spatial memory impairments (Ilieva et al., 2019), in the amyloid model this effect was not as clearly accounted for in the RAM test. Although we found similar numbers of neurons per unit area in STZ-veh/Aβ-veh groups in most brain structures examined, we can assume that the positive effect of Ago on spatial memory and cognitive abilities is due to synaptic remodeling phenomena influenced by it (De Berardis, Fornaro, Serroni, Campanella, Rapini, Olivieri, Srinivasan, Iasevoli, Tomasetti, De Bartolomeis, Valchera, Perna, Mazza, Nicola, et al., 2015). However, Ago has demonstrated neuroprotection in the CA1 subfield of the dorsal hippocampus as well as in the Pir, the two regions that are in close proximity and associated with cognitive function (Squire, 2009). And in the amyloid model, despite the reported

neuroprotective effect, the beneficial effect of the drug was not residual to improve hippocampal-dependent spatial memory.

In the amyloid model, we investigated the impact of two separate treatment approaches related to Ago treatment: during early and late stage AD progression. We found that although both treatment methods, AβAgo1 and AβAgo2, succeeded in preventing extracellular Aβ accumulation in the frontal cortex and hippocampus, there was a difference in terms of the effect on α-secretase concentration. Its content was found to be elevated with Aβ-Ago1 but not with Aβ- Ago2 treatment in the hippocampus, whereas both treatment conditions were able to significantly reduce Aβ levels and significantly reduce γ-secretase content in this brain region. Administration of Ago in early and late stage AD had no effect on β-secretase concentration, but at the same time was able to attenuate the icvAβ1-42-induced increase in γ-secretase levels in the hippocampus. These results suggest that both mechanisms may contribute to the beneficial effects of Ago in AD, but its application at earlier stages may be more beneficial. The hippocampus is known to be vulnerable and damaged in AD patients as it is responsible for short-term memory, which is significantly impaired. Long-term memory, primarily based in the frontal cortex, is not severely affected during the early stages of progression and much less so during the later stages (King,. 2007). While γ-secretase regulates the formation of Aβ1-40 and Aβ1-42 as a result of APP cleavage, α-secretase is not involved in the amyloidogenic pathway and the products of APP following its cleavage: APPαs or sAPP favor neuroplasticity and neuroprotection (Siegel et al., 2017). This finding is intriguing and requires further research to elucidate the exact mechanism by which Ago affects Aβ metabolism.

Despite the reported positive effects of Ago in behavioral and biochemical tests in both models, it did not exert strong neuroprotection in brain regions vulnerable to behavioral changes. The suggestion that he improved cognitive abilities was probably achieved by correcting synaptic communication without inducing neurogenesis has already been mentioned. In previous studies, we reported that Ago prevents neuronal loss in the dorsal hippocampus (CA1, CA2, CA3 field) and the gyrus dentatus hilus, where, however, it does not affect the decline in spatial memory in a kainate rat model of epilepsy (Tchekalarova et al., 2017). These results suggest a lack of a positive relationship between spatial memory and neuroprotection in the hippocampus, which may underlie the effectiveness of Ago in both models of sAD. The beneficial effect of Ago on memory impairments reflected in the streptozotocin model is logically related to its beneficial effect on cognitive functions. Like melatonin, Ago is characterized by a putative chronobiont activity (Tchekalarova et al., 2015). A limitation of the present study is that the effect of Ago was not also examined on a control group treated with Ago alone to confirm that it does not have a heterogeneous effect and to be able to make comparisons between sham-veh and sham-Ago groups. The lack of adverse effects or complications resulting from Ago treatment have been demonstrated in previous studies performed by our team (Tchekalarova et al., 2016). As early as 2011, in a study by (Morley-Fletcher et al., 2011), Ago was shown to affect mechanisms underlying anxiety/depressive disorders, which we have also analyzed in this dissertation. We conducted chronic Ago treatment in both models in the afternoon, when the chronobiotic activity of this drug, related to MT receptor activity, is predicted to be highest. Melatonin MT1 and MT2 receptors have been suggested to play different roles in AD, depending on the brain region affected. Thus, while their number is reduced in the cortex and pineal gland (Y. Li et al., 2020), MT1 receptors are increased and MT2 receptors are not expressed in the hippocampus (Klosen, 2024). The observed receptor plasticity in AD may be related to changes in melatonin levels, as the hormone has been shown to regulate MT receptor density (Feng et al., 2023). The reported neuropathological severity correlates with hippocampal-dependent spatial memory impairment as measured by the RAM

test, which is also consistent with previous experimental (Knezovic et al., 2015; Rudnitskaya et al., 2015) and clinical evidence of cognitive regression from mild cognitive impairment to progressive memory loss in AD (Sabbagh et al., 2013).

In conclusion, we can summarize that chronic treatment with Ago has a beneficial effect on comorbid behavioral impairments in the models of AD we studied and positively corrects levels of associated neurological markers. Treatment with Ago, initiated both during early and later stages of AD progression, had a beneficial effect on emotional disturbances and memory impairment by positively affecting Aβ metabolism through suppression of γ-secretase concentration, reduces tau protein deposition, reduces levels of pro-inflammatory signalling molecules in the frontal cortex and in the hippocampus and, last but not least, manages to correct the consequences of neuronal loss in some of the brain structures analysed. Future experimental and clinical studies are needed to establish whether the beneficial potential of this antidepressant in sAD induced in experimental animals is translatable to humans and would find application in clinic practice.

Conclusions

1. The streptozotocin model is characterized by increased expression of Aβ and tau-protein markers in frontal cortex and hippocampus three months after toxin infusion.

2. Agomelatine ameliorated the increased expression of Aβ and decreased the levels of neuroinflammatory markers and tau protein in frontal cortex and hippocampus in the streptozotocin model.

3. Chronic Ago treatment improved spatial memory in the streptozotocin model and reduced feelings of anxiety.

4. The rat model of AD induced by $A\beta_{1-42}$ is verified in the two brain structures studied two months after the infusion of the amyloid protein.

5. Treatment with Ago during the early stage of progression of AD, induced by $icvA\beta_{1-42,}$ alleviates disturbances in emotional status and reduces anhedonia.

6. Ago treatment at an early stage of progression in an Aβ model of AD exerts a protective effect selectively in the CA1 field of the dorsal hippocampus.

7. Both Ago treatment approaches, during both early and late stages of AD progression, positively affected Aβ metabolism by reducing elevated levels of γ-secretase in the hippocampus and increasing those of α-secretase in the frontal cortex and hippocampus.

Contributions

1. Original contributions:

1. In two rat models, induced by different toxins, it was found that treatment with Ago alleviated the cognitive impairment and increased anxiety characteristic of AD, which effect correlated with a decrease in Aβ levels in the hippocampus and evoked neuroprotection most pronounced in the CA1 field of this structure.

2. The beneficial effects of Ago in the streptozotocin model of AD are mediated by reduction of proinflammatory markers in frontal cortex and hippocampus.

3. In the amyloid model, changes in the amounts of secretory enzymes in the hippocampus under the influence of the agomelatine were detected for the first time.

2. Contributions of confirmatory nature

1. Neuronal loss in certain brain structures (hippocampus, basolateral amygdala and piriform cortex) characteristic of icvSTZ and Aβ1-42 infusion was confirmed.

2. The two rat models, icvSTZ and Aβ1-42, are characterized by increased Aβ expression.

3. The streptozotocin model showed increased levels of proinflammatory cytokines (IL-1β and TNF-α) and tau protein in frontal cortex and hippocampus.

Appendices

1. List of scientific publications

1. Ilieva K, Tchekalarova J, Atanasova D, Kortenska L, Atanasova M. Antidepressant agomelatine attenuates behavioral deficits and concomitant pathology observed in streptozotocin-induced model of Alzheimer's disease in male rats. Horm Behav. 2019;107. ISSN0018506X, https://doi.org/10.1016/j.yhbeh.2018.11.007

2. Ilieva K, Atanasova M, Atanasova D, Kortenska L, Tchekalarova J. Chronic agomelatine treatment alleviates icvAβ-induced anxiety and depressive-like behavior by affecting Aβ metabolism in the hippocampus in a rat model of Alzheimer's disease. Physiol Behav. 2021;239.DOI/10.1016/j.physbeh.2021.113525

3. Ilieva K, Tchekalarova J, Atanasova M, Petrova, L. Beneficial influence of agomelatine treatment on behavioral impairments in Aβ-induced rat model of Alzheimer's disease. Journal of Biomedical and Clinical Research 2020; 13(2), 116-121. (ISSN 1313-6917) http://jbcr.mu-pleven.bg/pdf/vol13no2/4.pdf

2. List of participations in scientific forums and conferences

1. Ilieva K, Tchekalarova J, Kortenska L, Mitreva R, Atanasova M, Chronic agomelatine treatment attenuates beta-amyloid accumulation, memory impairment and anhedonia in a ratmodel of sporadic Alzheimer's disease. AAT-AD/PD Focus meeting, Turin, Italy, 15-18 March 2018

2. Ilieva K, Cekalarova J, Atanasova D, Kortenskaya L, Atanasova M, Impact of Alzheimer's-related pathology in rat streptozotocin-induced models treated with agomelatine. Poster, XIII Scientific Conference on Medical Biology, Varna, 13-15.09.2019.

3. Ilieva K, Atanasova M, Chekalarova Y. Agomelatine provokes behavioral changes in two rat models of Alzheimer's disease. Poster, Scientific conference dedicated to the 100th anniversary of the birth of Professor Leon Mitrani. December 16, 2021. Institute of Neurobiology, ADS

4. Ilieva K, Atanasova M, Chekalarova J. Agomelatine affects the Aβ metabolism by adjusting the content of secretase enzymes in icv-Aβ induced rat model of Alzheimer's disease. XIV Scientific Conference on Medical Biology, Varna, 02-04.06.2023.

3. Realized projects on the topic of the dissertation

1. №7/2017 "Effects of the selective melatonergic drug agomelatine on the development of depressive-like behavior in a model of Alzheimer's disease (AD) in male Wistar rats." - MU-Pleven

2. №14/2018 "Effects of the selective melatonergic drug agomelatine on depressive behavior in two animal models of Alzheimer's disease." - MU-Pleven