Original article

Evaluation of neurobiological and antioxidant effects of novel melatonin analogs in mice

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ABSTRACT

Based on the pharmacophore model of melatonin (MT_1) receptor, we recently synthesized a series of indole derivatives that showed anticonvulsant activity with low neurotoxicity and hepatotoxicity in rodents. In the present study, the three most potent C3-modified derivatives with hydrazine structure **3c**, **3e**, and **3f**, with 2-chlorophenyl, 2-furyl, and 2-thienyl fragments, respectively, were selected, and their neurobiological activity was explored in mice. In *Experiment #1,* the dose-dependent anxiolytic effect of a single i.p. administration of the novel compounds at doses of 10, 30, and 60 mg/kg were studied in the open field (OF) test. In *Experiment#2,* the analgesic effect of **3c**, **3e**, and **3f** (30–100 mg/kg) was tested in the hot plate test and formalin test. *Experiment#3* was designed to assess the antidepressant-like activity of **3c**, **3e**, and **3f (**10–60 mg/kg). The forced swimming test (FST) and tail suspension test (TST)-induced effect on markers of oxidative stress in the frontal cortex (FC), and the hippocampus was evaluated. Melatonin was used in the same doses as melatonin analogs in all three experiments as a positive control. Desipramine (10 mg/kg) was also applied as a control in the FST. The three melatonin analogs bearing hydrazide/hydrazone substitution at 3C of the indol scaffold demonstrated improved antidepressant-like activity compared to the melatonin. The tested substances are devoided of anxiolytic effects. The antioxidant activity of the melatonin analogs and analgesic potential is comparable to that of melatonin. The 3C substitution with hydrazide/hydrazone moiety substantially contributes to the antidepressant and antioxidant activity of the melatonin analogs.

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1. Introduction

Depression is a severe, potentially life-threatening disorder that affects hundreds of millions of people all over the world. Dysfunctional neurotransmitter systems, including serotonin (5-HT) and norepinephrine (NE) in the central nervous system (CNS), as well as impaired balancein the endocrine and immune system, are hypothesized to underlie the pathophysiology of depression

(Lucas et al., 2010). The major disadvantage of classical antidepressants is related to their delayed therapeutic response, tolerability due to long-term usage, various side effects as well as drug-resistance, reported in >30% of patients (Fishback et al., 2010). Moreover, the precise underlying mechanism of antidepressant drugs is still not entirely clear. Therefore, the need for more efficient, faster, and with fewer side effects compounds for depression is imperative. Nowadays, the effort in developing potent antidepressants is expanded beyond standard treatments related to monoamine systems, and activities associated with the development of new therapeutic strategies have focused on other less well-characterized novel targets.

The melatonin, primarily secreted by the pineal gland in mammals, is a neurohormone with many different features, making it attractive for pharmacological research. Melatonin regulates the circadian and seasonal rhythms, controls the sleep cycles, and beneficially influences the depression-like behavior, anxiety, and

memory deficits (Hardeland, 2016; Hansen et al., 2014; Pandi-Perumal et al., 2008; Tchekalarova et al., 2015, 2019). It was also found that melatonin regulates the immune functions, possesses anti-inflammatory (Maestroni, 1993), anti-excitatory, neuroprotective and sedative effects (Hardeland et al., 2011). Due to its potential to influence crucial molecular mechanisms in the pathogenesis of depression, like monoamine level, oxidative stress, and chronic low-grade inflammation, melatonin was tested as a candidate-antidepressant (Hardeland, 2016; Hansen et al., 2014; Pandi-Perumal et al., 2008; Tchekalarova et al., 2015, 2019). However, despite the positive preclinical tests, it fails as antidepressants in humans, in the opposite of the melatonin structurally closely related agomelatine. Other investigators also report exogenous melatonin's analgesic activity in both animals and humans (Zhu et al., 2017). Due to the beneficial safety profile, anxiolytic, and analgesic activity, it is proposed adjuvant treatment in children with pain conditions (Marseglia et al., 2015). However, no one guideline proposes melatonin as analgesics, as far as we know.

In the difference of the many drugs in use, which are receptor antagonists, the melatonin pharmacology research is focused on agonist development (Boutin and Legros, 2020). Melatonin acts mainly via two G-protein coupled receptors, MT1 and MT2, but also, non-receptor melatonin binding sites are increasingly reported. (Emet et al., 2016). Thus, several different mechanisms were proposed for explanation of the antioxidant properties of melatonin, including binding with MT3-binding site and the cytosolic enzyme quinone reductase 2 (MT3/QR2) (Nosjean et al., 2000; Dubocovich et al., 2003), as well as direct free radicals scavenging (Tan et al., 2015). Due to this indoleamine's hydrophilic properties, melatonin reacts readily with intracellular targets as an electron donor. Moreover, in low pM and nM concentration, melatonin increases the activity or the expression of the enzymes superoxide dismutase, catalase, and glutathione peroxidase involved in the oxygen detoxification (Rosen et al., 2012),

Several hundred melatonin agonists were synthesized in recent decades; however, only a few are selective ligands for MT2. The MT1 selective ligands are either antagonists or partial agonists with significantly reduced selectivity in functional studies (Descamps-François et al., 2003). The search for MT1 or MT2 selective drugs with improved efficacy and a half-life more prolonged than that of melatonin fostered non-natural melatonin ligands development during the last decades (Gürkök et al., 2009).

Melatonin is an indole derivative bearing an N-acetyl-2-aminoethyl chain at C3 and a methoxy group at C5 (Fig. 1). The most-reported melatonin analogs are based on modification at N1, C2, or C3. Apart from the melatoninergic activity, some C3-modified melatonin analogs demonstrate high antioxidant, analgesic, and neuroprotective effects (Wang et al., 2020). Here we are reporting neuropharmacological activity of C3-modified

derivatives with hydrazine structure. Hydrazide/hydrazone derivative compounds are extensively investigated for their CNS-related effects, including antidepressant, analgesic, anticonvulsant, and other activities (Rollas and Küçükgüzel, 2007). The first generations of monoamine oxidase (MAO) inhibitors antidepressants, i.e., iproniazide, isocarboxazid, nialamide, etc. are hydrazine derivatives.

We recently reported a series of molecular hybrids bearing two highly promising pharmacophoric moieties, i.e., indole and aroyl-hydrazone, based on the melatonin receptor's (MT1) pharmacophore model (Angelova et al., 2019). Further, these novel compounds were evaluated *in vivo* for their potential anticonvulsant and neurotoxicity activity in mice and *in vitro* for potential hepatotoxicity in rats (Angelova et al., 2019; Marchev et al., 2019, Tchekalarova et al., 2019). Scientific rationale and perspectives for the synthesis of melatonin analogs bearing hydrazine-hydrazone scaffold in our work are to create synergistic neuropharmacological effects and to have carbonyl scavenger activity as well as antioxidant activity from hydrazine. The azomethine linkage of hydrazones influences the majority of their reactions and properties (Alisi et al., 2019). Three C3-modified derivatives with hydrazine structure, **3c, 3**e, and **3f** (with a 2-chlorophenyl 2-furyl, and 2-thienyl substituents respectively) were selected the most promising for further neuropharmacological testing (Fig. 1). In the present study, a dose-dependent anxiolytic, antidepressant, analgesic, and the stress-induced antioxidant effect was explored in a battery of tests in ICR mice. We hypothesized that the possible neurobiological activity of the compounds **3c, 3e**, and **3f** could be a background for the future design and elaboration of novel melatonin-related analogs as potential drugs.

2. Methods

2.1. Animals

All experiments were performed on naïve male ICR mice weighing 25–30 g, delivered from the animal facility of the Institute of Neurobiology, Bulgarian Academy of Sciences. The animals were maintained in the controlled environmental conditions (room temperature at 22 ± 1 °C, humidity 50 ± 5%; 12 h dark/light cycle) with food pellets and water *ad libitum* and in standard cages in groups of 10–12. After at least a week to accommodate in the laboratory conditions, the tests were performed between 10 a.m. and 1p.m. to avoid circadian influences. All procedures were performed in agreement with the European Communities Council Directive 2010/63/EU. The experiments were approved by the Bulgarian Food Safety Agency (license no. № 184/2017).
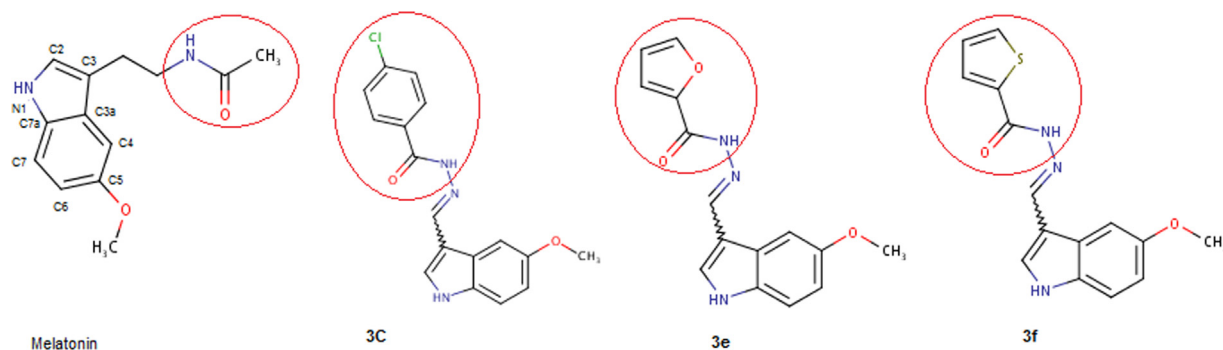

Fig. 1. Chemical structures of the melatonin and compound **3c**, **3e**, and **3f**.

2.2. Drugs

Melatonin and desipramine were purchased from Merck KGaA, Darmstadt, Germany. The three tested substances were synthesized in the Chemistry Department of the Faculty of Pharmacy of the Medical University of Sofia. Their synthesis and spectra are already published elsewhere (Angelova et al., 2019).

2.3. Experimental design and drug administration

The neurobiological activity of the compounds **3c**, **3e**, and **3f** was evaluated in three experimental protocols where a separate cohort of animals was used. *Experiment#1* was designed to assess the dose-dependent effect on motor activity and anxiety of a single administration of **3c**, **3e**, and **3f**. The mice received intraperitoneal (i.p.) injection of vehicle (control, C), melatonin, and the novel compounds at doses of 10, 30, and 60 mg/kg, respectively, dissolved in 10% dimethyl sulfoxide (DMSO) in saline solution 0.5 h before the open field (OF) test. The volume of administered solutions/suspension was 0.1 ml per 10 g of body weight. In *Experiment#2*, the dose-dependent analgesic effect of **3c**, **3e**, and **3f**, administered at 30, 60, and 100 mg/kg, respectively, in mice was explored 0.5 h after injection in the hot plate test or formalin test, respectively. In *Experiment#3*, the dose-dependent antidepressant effect was assessed. The treatment with these compounds was as in the *Experiment#1*. The stress-induced markers of oxidative stress in the frontal cortex (FC) and the hippocampus of melatonin, **3c**, **3e**, and **3f** were explored at a dose of 60 mg/kg. Mice were tested in the forced swimming test (FST). A separate group of mice was tested in a tail suspension test (TST). Five minutes later, the mice were decapitated under mild anesthesia with CO_2. After a rapid dissection of brains on ice, the frontal cortex (FC) and the hippocampi were bilaterally isolated. The tissue samples were immediately frozen in liquid nitrogen and stored at −70 °C before biochemical analysis. Matched control groups without exposure to TST and injected with vehicle, melatonin, or the compounds **3c**, **3e**, and **3f**, respectively, were used for oxidative stress measurement. The size in each group in *Experiment# 1, 2,* and *3* consisted of six to eight mice per group. The doses and time interval (the time-to-peak effect) were chosen based on our previous reports (Angelova et al., 2019; Marchev et al., 2019; Tchekalarova et al., 2019). Animals from the control groups received i.p. injection of vehicle.

2.4. Test for anxiety

The anxiety was examined in the open field test. The tested mouse was gently placed at the central area of a grey polystyrene box (50 × 50 cm × 40 cm) for 5 min. The total distance traveled (cm), a number of rears (vertical activity), the length (cm) moved, and time spent in the aversive central zone of the apparatus was detected automatically by a video tracking system (SMART PanLab software, Harvard Apparatus, USA).

2.5. Tests for analgesia

Before the test, mice underwent overnight fasting but had free access to water. Each animal was tested only in one test.

2.5.1. Hot plate test

Twenty-four hours before testing, all mice were given 5 min to acclimate to the hot plate. Acute thermal pain was induced using a temperature of 47 °C. Tested mice were placed in an open-ended cylinder over the hot plate equipped with a thermostat (Ugo Basile, Italy) and observed for two types of reaction, that is paw licking and jumping. The latent period for the antinociceptive behavior of mice was measured (licking of the hind paws or jumping).

2.5.2. Formalin test

This test in mice is indicative of continuous pain. Subdermal injection (left posterior paw) of 1% 0.02 ml formalin solution was applied 30 min after the mice were injected i.p. with the test compounds or with the vehicle. The animals were observed for 40 min in total to detect a typical two-phase reaction. During the first phase (0–5 min), the time (in seconds) for the licking of the paw was recorded. After a latent period, the second phase of antinociceptive behavior (between the 20th and 30th min) was observed, and the time in seconds was recorded manually.

2.6. Tests for a screening of antidepressants

2.6.1. Forced swimming test

FST was executed following the protocol of Porsolt et al. (1977a). In brief, the mouse was placed into a clean glass cylinder (height 25 cm, diameter 10 cm) filled up to 15 cm of water at 23–25 °C for 6 min. After the first 2 min of the test, which was not indicated, the total duration of immobility (sec) was measured. The immobile state of the animal was accepted when it has stayed motionless and attempted only to keep the head above the surface of the water without struggling.

2.6.2. Tail suspension test

The test was performed following the method described by Steru et al., 1985. The mouse was gently suspended by the tail (about 2 cm from the end of the tail) via adhesive tape for 6 min. As in the FST, the first 2 min of the test was not indicated, and the total duration of immobility (sec) was measured during the next 4 min. A pose without moving limbs and body was accepted as the immobility behavior of the experimented animal.

The two tests for depression, FST, and TST, were video recorded and then analyzed independently by two skilled and experimenters.

2.7. Detection of oxidative stress in mouse homogenates

The oxidative stress was evaluated by the assessment of the level of lipid peroxidation, expressed as of malondialdehyde (MDA), and by the activity of two antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx). Two brain structures, the frontal cortex (FC) and the hippocampus from mice (n = 8) were harvested and homogenized mechanically in PBS (Phosphate Buffered Saline, pH 7.4) containing protease inhibitors (Thermo Fisher Scientific) in ratio 9 ml PBS/1 g tissue.

For detection of oxidative stress were used Mouse Gpx1 (Glutathione peroxidase 1) ELISA kit (Wuhan Fine Biotech Co., Ltd., Wuhan, China, Cat. No: EM0367); Mouse SOD1 (Superoxide dismutase [Cu-Zn]) ELISA kit (Wuhan Fine Biotech Co., Ltd., Wuhan, China, Cat. No: EM0419) and MDA ELISA kit (Wuhan Fine Biotech Co., Ltd., Wuhan, China, Cat. No: EU2577) following the manufacturer's instructions for the assay procedure. The obtained absorbance was read at 450 nm with the 96-well plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). The results were calculated using a professional software curve expert (MyCurveFit, Online Curve Fitting, http://www.fn-test.com/services/software-download/).

2.8. Statistical analysis

All data were assessed for equality of variance and normality of distribution and are presented either as mean ± standard error (x ± S.E.M.) or as median and interquartile ranges. For each treat-

ment group, i.e., melatonin, **3c**, **3e**, and **3f**, respectively, the results were analyzed by a separate one-way ANOVA followed by Dunnett's *post hoc* test to compare the differences among control and treatment group administered in three different doses. Data of oxidative stress were analyzed by two-way ANOVA with factors Stress and Treatment followed by Bonferroni *post hoc* test if a main factor effect or interaction between the two factors was indicated. In case of lack of homogeneity, a non-parametric analysis (Kruskal-Wallis on ranks) followed by Dunnett test or the Mann-Whitney *U* test was applied, and in this case, the figures were given as scattered plots. Parametric data were presented as scattered plots with bars. A p < 0.05 value was accepted as a statistically significant difference. Figures were prepared with box plots Figures, calculations, and statistical analyses were performed with Graph Pad Prizm version 7.04 (GraphPad Software, San Diego, CA, USA) and SigmaStat® 11.0 (Systat Software Inc., San Rose, USA).

3. Results

3.1. Experiment#1

3.1.1. Effects of melatonin and melatonin analogs, 3c, 3e, 3f on motor activity and anxiety measured in the open field test

Data from the total distance traveled, and the number of rears shows that neither melatonin nor any of the three compounds **3c**, **3e**, and **3f**, administered at doses of 10, 30, and 60 mg/kg, respectively, changed the motor activity (horizontal) (P > 0.05) (Fig. 2A, B, C, D) and (vertical) (P > 0.005 compared to control group) (Fig. 3A, B, C, D), respectively.

Overall, melatonin and the three melatonin analogs **3c**, **3e**, and **3f**, at the three doses tested, did not affect the anxiety level, measured by the distance traveled (P > 0.05 compared to control group)

(Fig. 4A, B, C, D) and the time spent in the aversive central zone (P > 0.05 compared to control group) (Fig. 5A, B, C, D).

3.2. Experiment#2

3.2.1. Effects of melatonin and melatonin analogs, 3c, 3e, 3f on nociception measured in the hot plate test and formalin test in phase i (0–5 min) and phase II (20–30 min), respectively

Data from the latency for antinociceptive behavior in the hot plate apparatus showed no significant difference between each treatment group i.e., melatonin, **3c**, and **3f** and control group [One-way ANOVA: P > 0.05] (Fig. 6A, B, D). However, one-way ANOVA demonstrated a significant effect for the treatment with the compound **3e** [$F_{3,23} = 4.016$, P = 0.022]. The *post hoc* test indicated that the compound with a 2-furyl fragment exacerbated nociception at the highest dose of 100 mg/kg used (P = 0.038 compared to the control group) (Fig. 6C).

Injection of 1% of formalin solution induced painful stereotyped behavior in two phases, an early phase, and a late phase, respectively, separated by quiet interphase. To assess both of the phases, we measured the painful behavior between 0 and 5 min (1st phase) and 20 and 30 min (2nd phase). In phase-1 (0–5 min), the differences in the mean values among the melatonin treatment groups were greater than would be expected by chance [Kruskal-Wallis test on ranks: H = 18.505, P < 0.001] as well as treatment groups with the compound **3c** [One-way ANOVA: $F_{3,23} = 3.656$, P = 0.03]. The *post hoc* test revealed that melatonin caused antinociceptive behavior only at the highest dose of 100 mg/kg (P = 0.002 compared to control) (Fig. 7A). The compound **3c** also alleviated the nociceptive response at a lower dose of 60 mg/kg than melatonin (P = 0.045 compared to control) (Fig. 7B). The compounds **3e** and **3f** did not affect the painful stereotyped behavior in
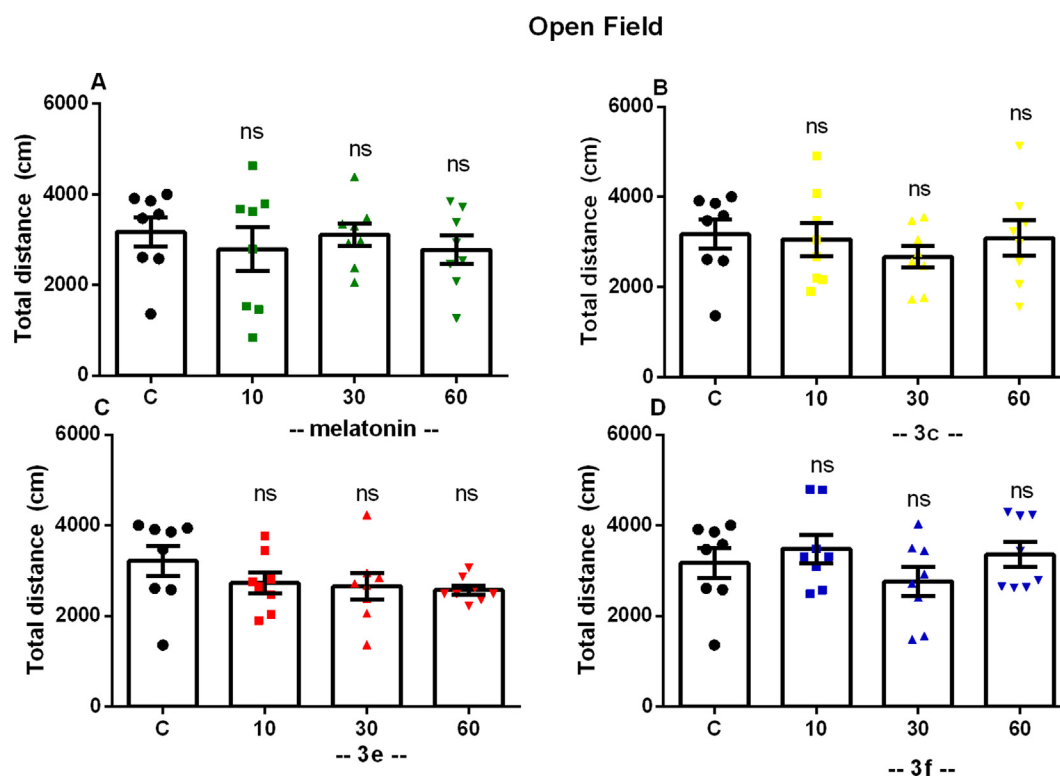

Fig. 2. Effects of melatonin (A), and the compound **3c** (B), **3e** (C), and **3f** (D) on motor activity, measured as horizontal activity (total distance, cm) in the open field (OF) test of ICR mice (n = 8). The drugs were administered 30 min before the test at doses of 10, 30 and 60 mg/kg, i.p. Control animals received 10% DMSO in saline solution. One-way ANOVA test was applied for A,B,C,D and the mean ± S.E.M of data were presented as scattered bars.
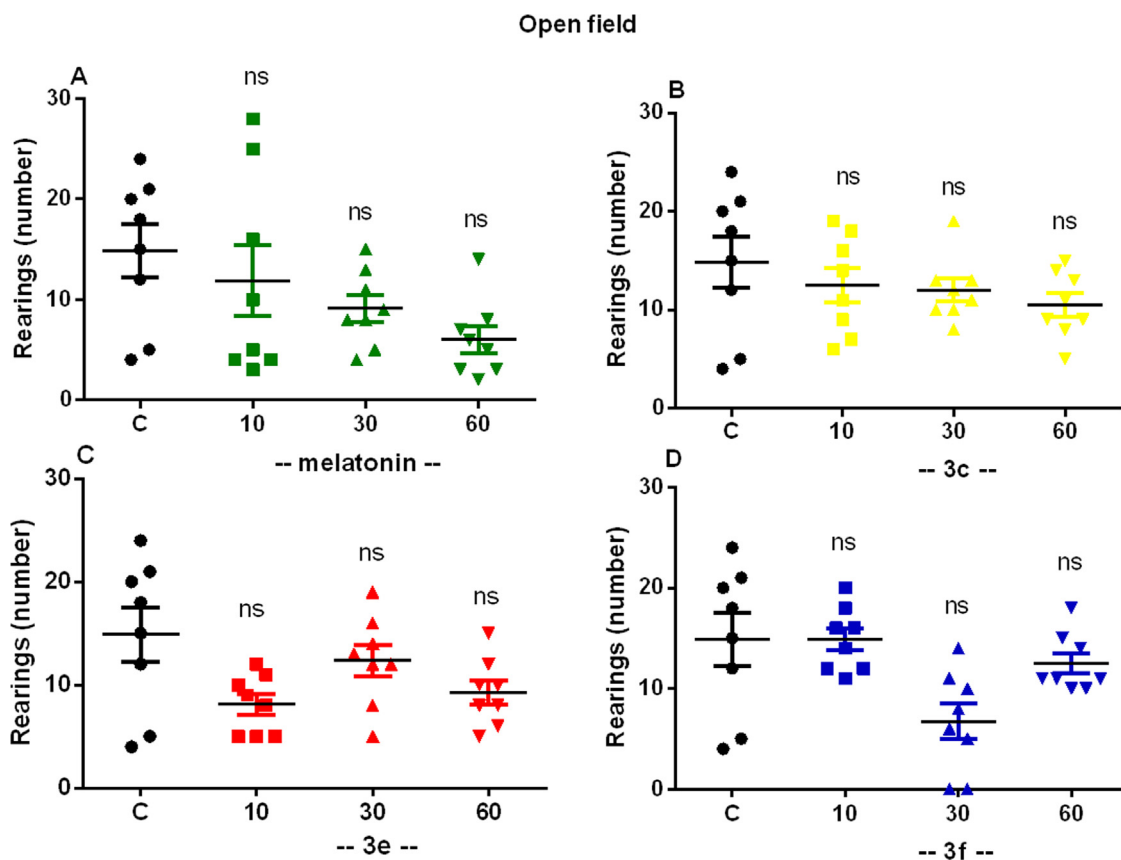
Open field

Fig. 3. Effects of melatonin (A), and the compound **3c** (B), **3e** (C), and **3f** (D) on vertical activity (number of rears) in the OF test of mice (n = 8). Kruskal-Wallis on ranks was applied for all data that were presented as scattered plots. The details are as in Fig. 1.

the early phase (0–5) used at the three doses of 30, 60 and 100 mg/kg, respectively (P > 0.05 compared to control) (Fig. 7C, D).

Data for the late phase II (20–30) of formalin test demonstrated that the differences in the median values among melatonin treatment groups were greater than would be expected by chance [Kruskal-Wallis on ranks: H = 16.162, P < 0.001]. Melatonin produced a dose-dependent analgesic effect, at doses of 30 mg/kg and 100 mg/kg, respectively, compared to the control group (P = 0.013 and P = 0.0002, respectively) (Fig. 7E). The three melatonin analogues also alleviated the behavioral response to the algogenic stimulus as follows: **3c** [Kruskal-Wallis on ranks**:** H = 18.353, P < 0.001]; **3e:** [One-way ANOVA: $F_{3,23}$ = 14.187, P < 0.001] and **3f:** [Kruskal-Wallis on ranks: H = 16.612, P < 0.001]. The *post hoc* test showed antinociceptive response of the three compound at doses of 60 mg/kg and 100 mg/kg, respectively: **3c** (P = 0.006 and P = 0.002 compared to the control group) (Fig. 7F), **3e** (P < 0.001 and P = 0.005 compared to the control group) (Fig. 7G) and **3f** (P = 0.01 and P = 0.002 compared to the control group (Fig. 7H).

3.3. Experiment#3

3.3.1. Effects of melatonin and melatonin analogs, 3c, 3e, 3f on depressive responses measured in the forced swimming test and tail suspension test stress-induced effect on markers of oxidative stress in the frontal cortex and the hippocampus

Data from FST demonstrated that melatonin positively affected despair-like response [Kruskal-Wallis on ranks: H = 20.728, P < 0.001]. The lowest dose of 10 mg/kg melatonin showed antidepressant effect comparible to the referent drug desipramine injected at the same dose (P < 0.001 compared to control) (Fig. 8A). The immobility behavior was also dose-dependently alle-

viated by the three compound as follows: **3c** [One-way ANOVA: $F_{3,31}$ = 10.784, P < 0.001], **3e** [Kruskal-Wallis on ranks: H = 19.681, P < 0.001] and **3f** [Kruskal-Wallis on ranks: H = 19.681, P < 0.001]. The *post hoc* test demonstrated antidepressant-like effect of the compound with 2-chlorophenyl fragment at the lowest dose of 10 mg/kg (P = 0.01 compared to control group), with 2-furyl group, at doses of 10 and 60 mg/kg, respectively (P < 0.001 compared to control group) (Fig. 8B, C). The compound with the 2-thienyl fragment showed dose-dependent activity detected at the doses of 10, 30 and 60 mg/kg (P = 0.013; P < 0.001 and P = 0.006 compared to control group) (Fig. 8A, B, C, D)

While, melatonin, was ineffective at the three doses used in the TST (P > 0.05 compared to control group), a significant treatment effect for melatonin analogs, administered at three doses, was demonstrated as follows: **3c** [One-way ANOVA: $F_{3,31}$ = 7.139, P < 0.001], **3e** [Kruskal-Wallis on ranks: H = 15.842, P = 0.001], **3f** [One-way ANOVA: $F_{3,31}$ = 4.369, P = 0.012]. The *post hoc* test confirmed that immobility time was positively affected by the compound **3c** and **3e** administered at a dose of 60 mg/kg (P = 0.048 and P = 0.001 compared to control group**,** respectively) and the compound **3f** at a dose of 30 mg/kg (P = 0.009 compared to the control group) (Fig. 9A, B, C, D).

Two-way ANOVA demonstrated a main effect of Stress [$F_{1,23}$ = 22.724, P < 0.001] and melatonin treatment [$F_{1,23}$ = 6.381, P = 0.02] for SOD activity in the FC without Stress × Treatment interaction. The stress-induced decrease in the SOD activity was detected in the vehicle-treated mice (P = 0.008) (Fig. 10A compared to C-veh group). Although melatonin alleviated the stress-induced decrease of SOD activity (P = 0.048 compared to TST-veh group), it was lower compared to C-mel group (P = 0.006).
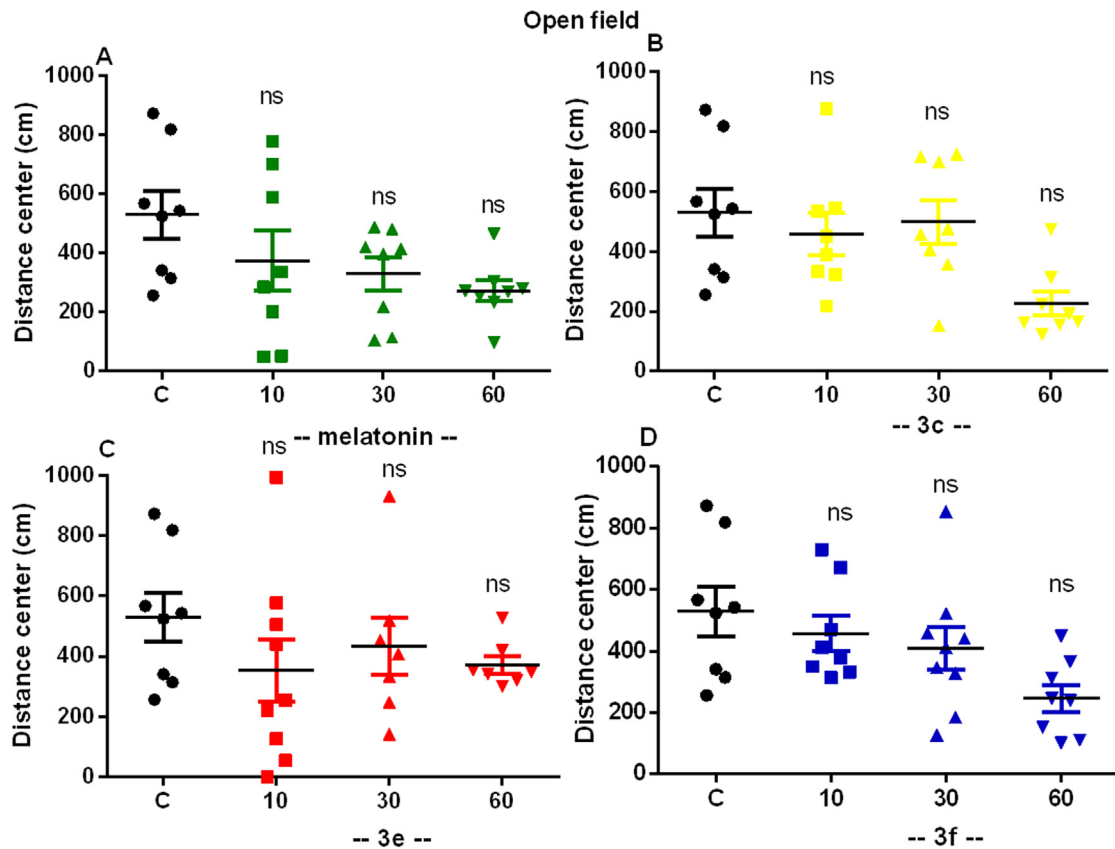
Fig. 4. Effects of melatonin (A), and the compound **3c** (B), **3e** (C), and **3f** (D) on distance traveled (cm) in the central aversive are in the OF test of mice (n = 8). The details are as in Figs. 1 and 2.

A significant Stress × Treatment interaction was detected for the SOD activity in the FC of both the compound **3c** [$F_{1,23}$ = 9.278, P = 0.005] and the compound **3f** [$F_{1,31}$ = 13.039, P = 0.002], respectively. The *post hoc* test confirmed that single injection of melatonin analogues with 2-chlorophenyl fragment and the 2-thienyl fragment, respectively, enhanced the activity of the antioxidant enzyme in the FC (P = 0.036 compared to veh-TST group) and (P = 0.009 compared to veh-TST group) (Fig. 10B, D).

Two-way ANOVA revealed a significant main effect of melatonin treatment in the hippocampus [$F_{1,23}$ = 8.881, P = 0.007], whereas the three melatonin analogs showed no activity (Fig. 11A, B, C, D). The *post hoc* test confirmed that the TST-mel group significantly elevated the enzyme activity (P = 0.046 compared to TST-veh group) (Fig. 11A).

Two-way ANOVA demonstrated a main effect of melatonin treatment for the GPx activity in the FC [$F_{1,23}$ = 11.280, P = 0.004] (Fig. 12A). The *post hoc* test showed that melatonin enhanced the GPx activity of the stressed group (P = 0.007 compared to the stressed control group) (Fig. 12A). The three melatonin analogs did not affect the GPx activity in this brain structure (Fig. 12B, C, D).

The main melatonin treatment effect was demonstrated for GPx activity in the hippocampus [$F_{1,23}$ = 6.632, P = 0.006]. The *post hoc* test showed that melatonin treatment enhanced the enzyme activity in the naive group (P = 0.0081 compared to the veh-C group) (Fig. 13A). Like in the FC, the compounds **3c**, **3e** and **3f** did not affect the GPx activity in the hippocampus (P > 0.05) (Fig. 13B, C, D).

Two-way ANOVA showed a significant main Stress effect as well as Stress × melatonin treatment interaction for MDA level in the FC [$F_{1,23}$ = 34.059, P < 0.001]. *The post hoc* test revealed stress-induced

elevation of the lipid peroxidation in the FC of vehicle-treated group (P = 0.01 veh-TST compared to veh-C group) while melatonin treatment alleviated stress-induced increased MDA level in the FC (P = 0.0085 compared to veh-TST group) (Fig. 14A). Further, Stress × Treatment interaction for MDA level in the FC was also detected for the compounds **3c** [$F_{1,23}$ = 11.047, P = 0.003], **3e** [$F_{1,23}$ = 20.976, P < 0.001], and **3f** [$F_{1,23}$ = 56.329, P < 0.001]. The three melatonin analogs alleviated the stress-induced increased MDA level in the FC (P = 0.007 **3c**-TST compared to veh-TST group; P = 0.001 **3e**-TST compared to veh-TST; P = 0.001 **3f**-TST compared to veh-TST) (Fig. 14B, C, D). However, the compound **3f** elevated the MDA level in the FC of naive mice (P = 0.043 compared to veh-C group) (Fig. 14D).

TST stress did not affect the MDA level in the hippocampus, and melatonin and its three analogs showed no effect on lipid peroxidation in this brain structure (P > 0.005 compared to the veh-TST group) (Fig. 15 A, B, C, D).

4. Discussion

4.1. Antidepressant-like effects of the tested substances devoid of anxiolytic-like activity

To assess the potential to suppress anxiolytic and depression-like activity, OF, TST and FST were used (Experiments #1 and #3). It is essential to mention that these tests only reproduce some aspects of the depressive symptoms characteristic for humans' depression (Valvassori et al., 2013). Neither melatonin nor the three melatonin analogs **3c**, **3e**, or **3f** demonstrated qualitative or quantitative changes in the measurement of exploratory and loco-motor activity and anxiety-like behavior in rodents in the OF test.
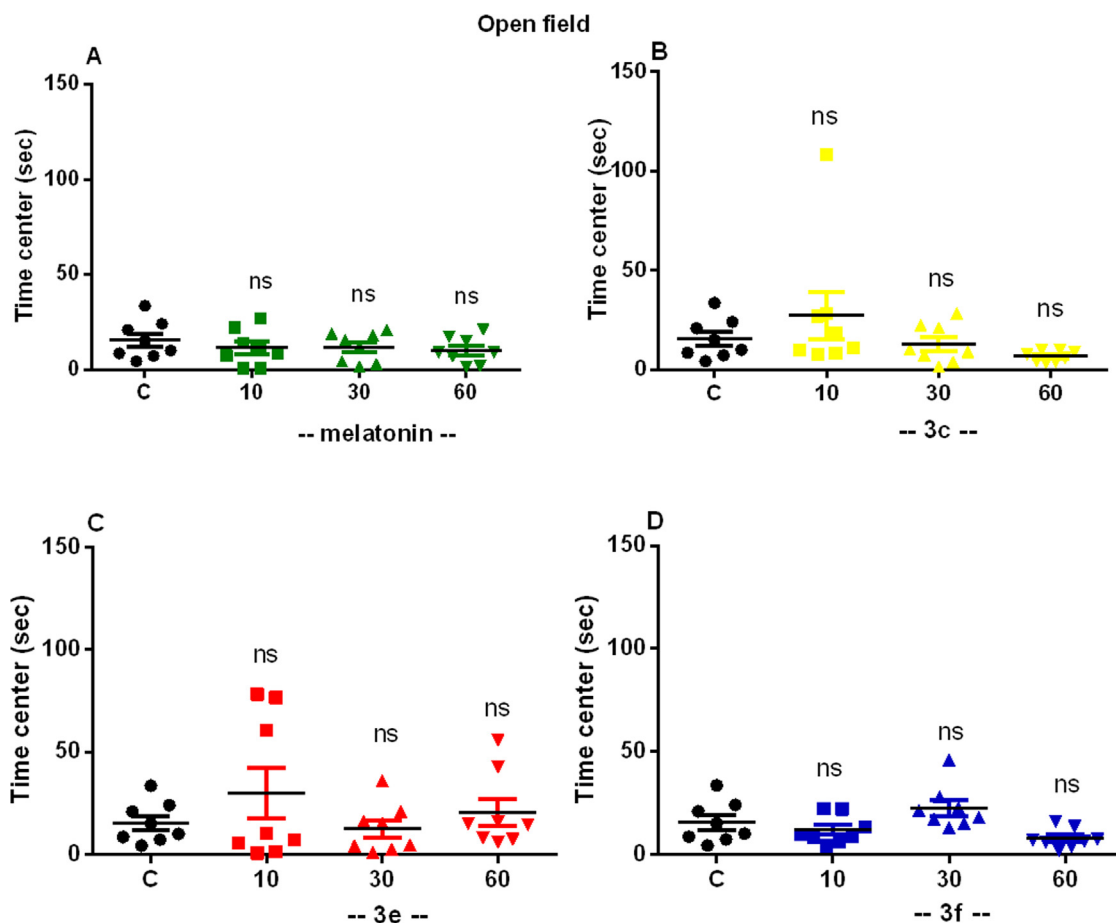
Open field

Fig. 5. Effects of melatonin (A), and the compound **3c** (B), **3e** (C), and **3f** (D) on time spent (sec) in the central aversive are in the OF test of mice (n = 8). Kruskal-Wallis on ranks was applied for all data that were presented as scattered plots. The details are as in Figs. 1 and 2.

Immobility in the FST has been interpreted as a manifestation of negative mood, a kind of hopelessness in the animal, corresponding to lack of motivation (Porsolt et al., 1977b). Melatonin and the three tested melatonin derivatives demonstrated comparable antidepressant-like activity in the FST. The tail suspension induces a state of immobility, which is interpreted as despair or helpless behavior in response to an inescapable and confined space (Yin et al., 2011). While melatonin failed to demonstrate antidepressant-like activity in the TST, the three melatonin analogs were active. We propose that their molecules' hydrazide/hydrazone moiety increase the antidepressant activity, possibly by MAO inhibition. It is typical for MAO inhibitors to show no anxiolytic-like effects in experimental models of anxiety (Gökhan-Kelekçi et al., 2009).

The three tested melatonin analogs **3c**, **3e**, and **3f** already demonstrated promising neuropharmacological activity against ivPTZ-induced seizures. The docking analysis suggested that the MT_1 receptors might be considered a potential target of the three melatonin analogs (Tchekalarova et al., 2019). Experimental and clinical data indicate the modest impact of melatonin on anxiety and depression behavior, which is strictly dependent on the time of delivery explained with its putative chronotropic activity and the crucial role of the endogenous hormone on circadian rhythmic processes (Tchekalarova et al., 2015). Here, we succeed to demonstrate that a 3C replacement with hydrazide/hydrazone moiety may increase the antidepressant potential of the molecules. Indeed, the three indole compounds exerted dose-dependent antidepressant activity in the FST and TST, while melatonin was

effective only in the FST. We performed the behavioral tests between 10 a.m. and 1p.m. when evidence suggests a melatonin's weak activity. We did perform experiments at the same time points to exclude the time as a crucial factor for the potency of the newly synthesized melatonin analogs. Such experiments are essential to be planned in the future to ascertain whether the effect of the new melatonin analogs is not affected by the time of exposure. We have evaluated only male mice to exclude the sex differences in the results.

4.2. Analgesic activity of the tested substances

Many antidepressants, i.e., tricyclic, gabapentenoids, others, are used in practice to treat chronic pain. Therefore, it is not uncommon to test the new neuroactive compounds for analgesic activity. *Experiment #2* was designed to compare the three new melatonin derivatives' effects versus melatonin's analgesia. Exception for the **3e** compound, which produced nociceptive effect at the highest dose used in the HP test, all melatonin analogs exerted analgesic activity in the second phase of the formalin test comparable to that of the positive control melatonin.

Other authors already reported that melatonin exerts an analgesic effect when applied to rodents at higher doses (60 mg/kg and 120 mg/kg). The effect was described to starts 15 min after melatonin administration and reached a peak after 30 min, with the effect lasting over 100 min (Yu et al., 2000). In diabetic rats, the best antinociceptive effect was observed when melatonin was applied in a dose of 300 mg/kg orally 60 min before the forma-
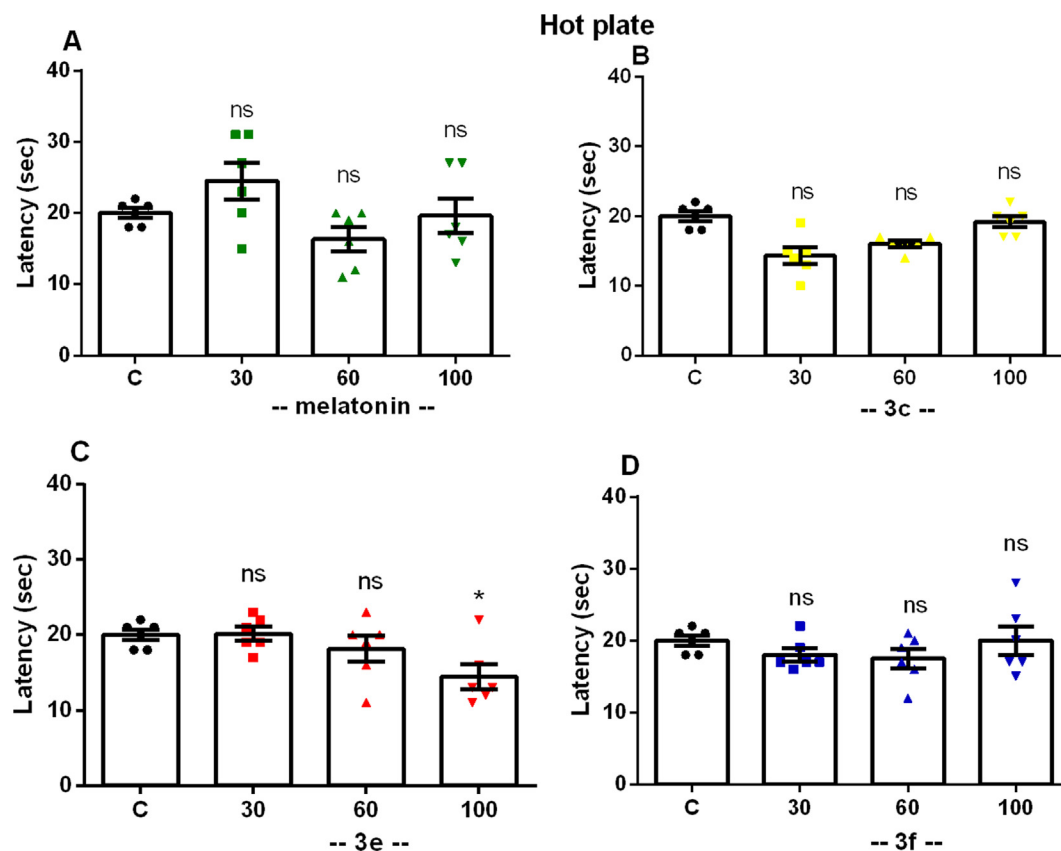
Fig. 6. Effects of melatonin (A), and the compound **3c** (B), **3e** (C), and **3f** (D) on latency from the hot plate exposure to the antinociceptive behavior (sec) measured in the hot plate test in mice (n = 6). The details are as in Fig. 1. (One-way ANOVA: ^{o}P = 0.038 TST-3c (100 mg/kg) group compared to veh-TCT group).
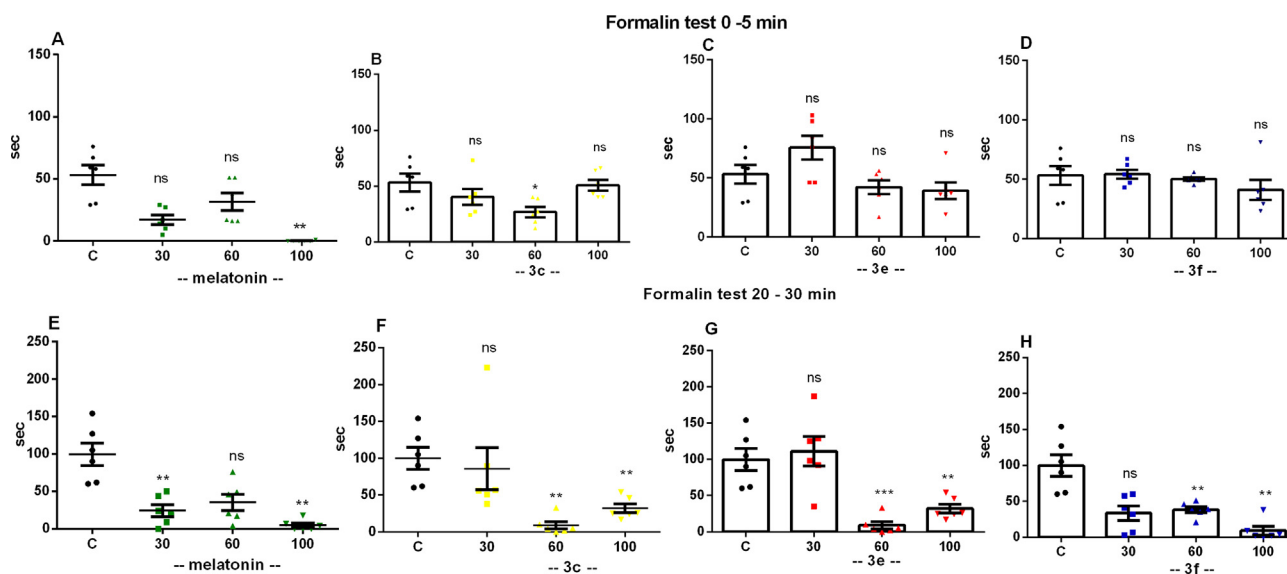

Fig. 7. The antinociceptive behavior (sec) during the Formalin test phase I (5–10 min): melatonin (A), **3c** (B), **3e** (C), and **3f** (D) and phase II (20–30 min): melatonin (E), **3c** (F), **3e** (G), and **3f** (H) in mice (n = 8). Kruskal-Wallis on ranks was applied for A, E, F data presented as scattered plots and one-way ANOVA for B,C,D,G,H presented as scattered boxes. **P = 0.002 melatonin (100 mg/kg) compared to control (A), *P = 0.045 **3c** compared to control (B), *P = 0.013 melatonin (30 mg/kg) compared to control (E), ***P = 0.0002 melatonin (100 mg/kg) compared to control (E), **P = 0.006 **3c** (60 mg/kg) compared to control (F), **P = 0.002 **3c** (100 mg/kg) compared to control (F), ***P = 0.001 **3e** (60 mg/kg) compared to control (G), **P = 0.005 **3e** (100 mg/kg) compared to control (G), **P = 0.01 **3f** (60 mg/kg) compared to control (H), **P = 0.002 **3f** (100 mg/kg) compared to control (H).

lin test (Arreola-Espino et al., 2007). Our finding of the melatonin's analgesic effect is in line with the literature. The hot plate test is expected to identify centrally acting analgesics (Laste et al.,

2012). The two phases of the formalin test may have different nociceptive mechanisms. The 1st phase is attributed to the direct nociceptive activity, whereas the second phase is related to
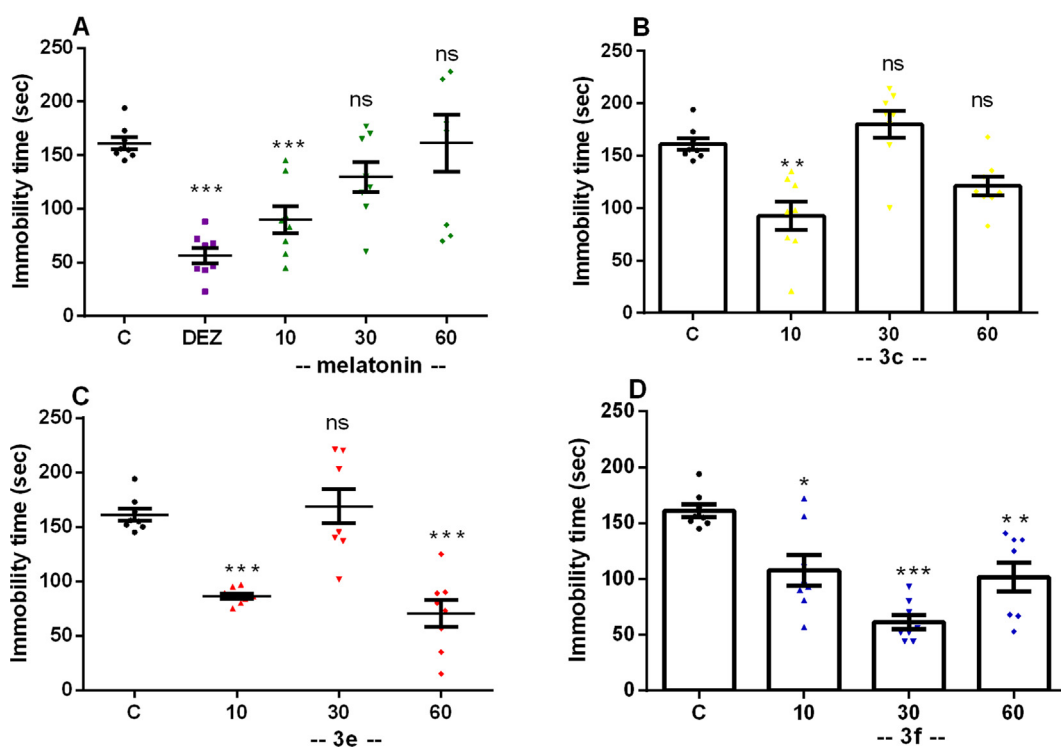
Forced swimming test

Fig. 8. Effects of melatonin (A), and the compound **3c** (B), **3e** (C), and **3f** (D) on immobility time (sec) measured in the forced swimming test (FST) in mice (n = 8). The details are as in Figs. 1 and 2. Kruskal-Wallis on ranks was applied for (A) ***P < 0.001 desipramine (10 mg/kg) compared to control group; ***P = 0.0007 melatonin (10 mg/kg) compared to control group; One-way ANOVA: **P = 0.001 **3c** (10 mg/kg) compared to the control group (B), Kruskal-Wallis on ranks: ***P < 0.001 **3e** (30 and 60 mg/kg) compared to control group (C), One-way ANOVA: *P = 0.013, ***P = 0.001 and **P = 0.006 **3f** (10, 30 and 60 mg/kg) compared to control group (D).
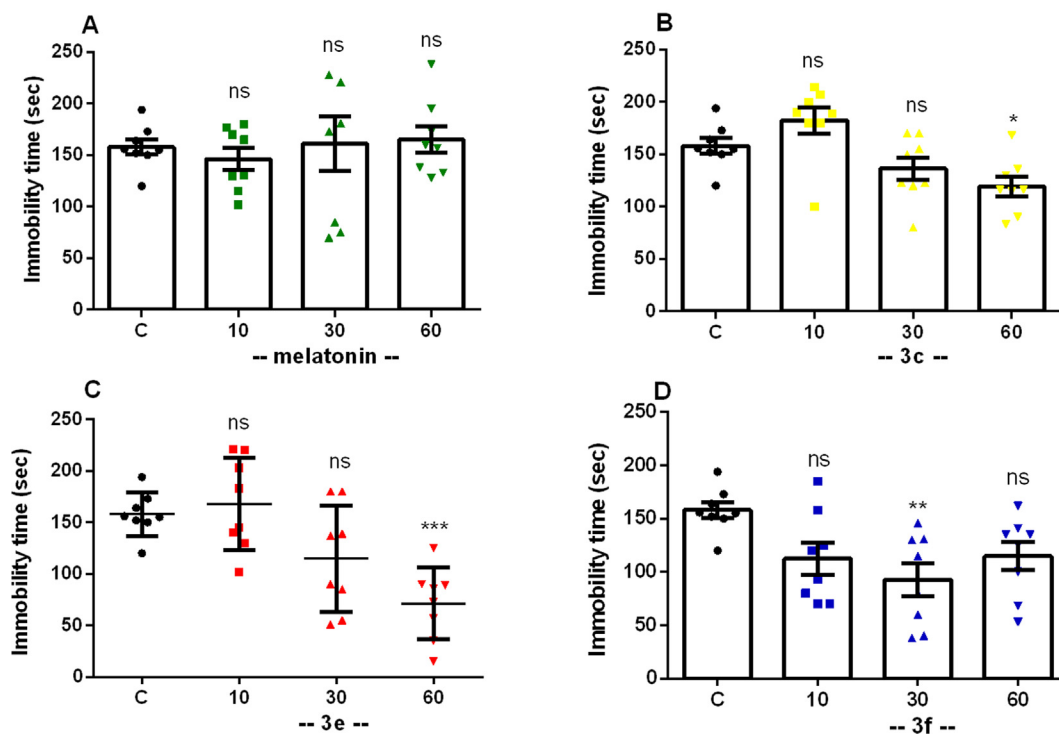
Tail suspension test

Fig. 9. Effects of melatonin (A), and the compound **3c** (B), **3e** (C), and **3f** (D) on immobility time (sec) measured in tail suspension test (TST) in mice (n = 8). The details are as in Figs. 1 and 2. One-way ANOVA:*P = 0.048 **3c** (60 mg/kg) compared to the control group (B), Kruskal-Wallis on ranks: **P = 0.001 **3e** (60 mg/kg) compared to control group (C), One-way ANOVA:**P = 0.009 **3f** (30 mg/kg) compared to control group (D).
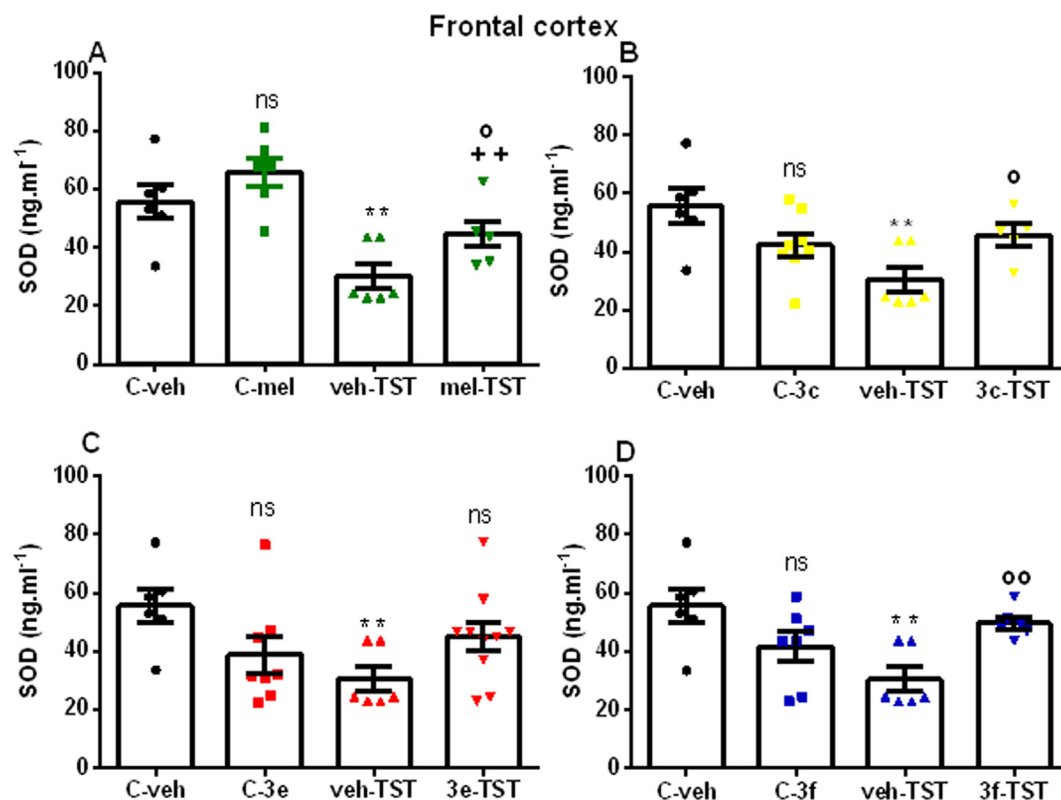
Fig. 10. Effects of melatonin (A) and the compound **3c** (B), **3e** (C), and **3f** (D) on superoxide dismutase activity (SOD) (ng/ml) measured in the frontal cortex (FC) in naive mice (C-mel, C-**3c,** C-**3e** and C-**3f**) and stressed by TST mice (mel-TST, **3c**-TST**, 3e**-TST and **3f**-TST) (n = 6). Two-way ANOVA was applied with Bonferroni post hoc test in case of statistical difference. (A,B,C,D) $^{**}P = 0.008$ veh-TST compared C-veh group; $^{o}P = 0.048$ compared to TST-veh group, $^{++}P = 0.006$ mel-TST compared to C-mel group (A), $^{o}P = 0.036$ **3c**-TST compared to veh-TST group (B); $^{oo}P = 0.009$ **3f**-TST compared to veh-TST group (D).
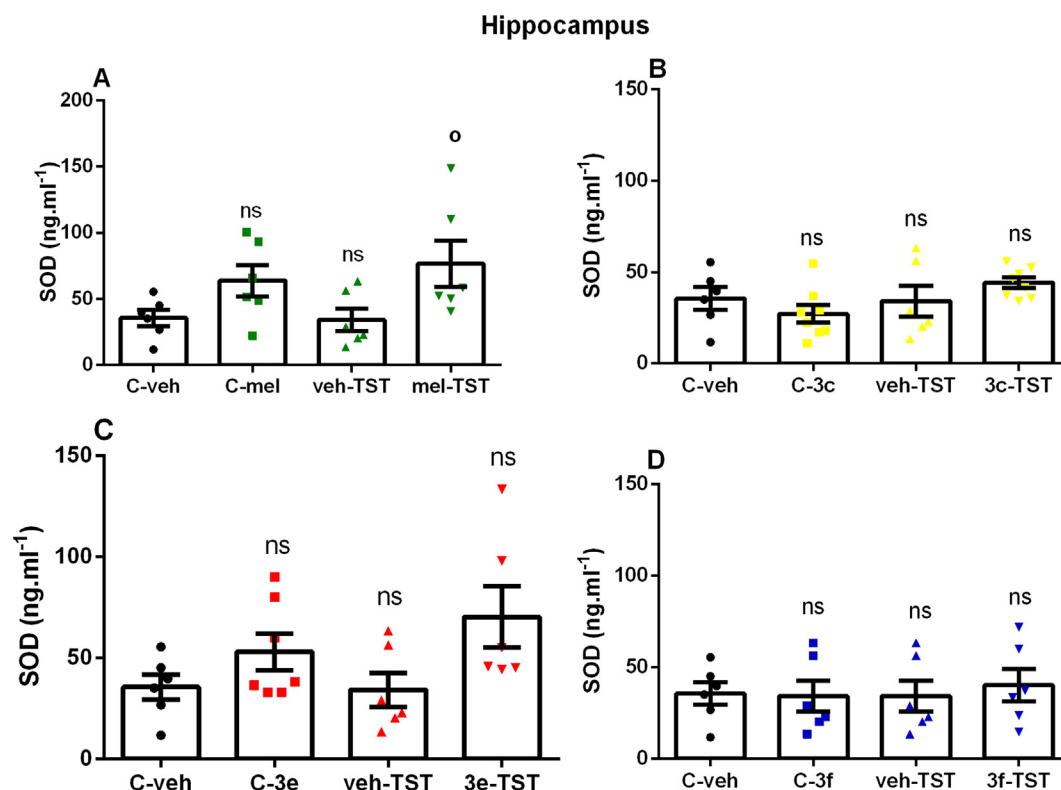

Fig. 11. Effects of melatonin (A) and the compound **3c** (B), **3e** (C), and **3f** (D) on SOD activity (ng/ml) measured in the hippocampus in naïve mice (C-mel, C-**3c,** C-**3e** and C-**3f**) and stressed by TST mice (mel-TST, **3c**-TST**, 3e**-TST and **3f**-TST) (n = 6). Details as in Fig. 10. $^{o}P = 0.046$ TST-melatonin compared veh-TST group (A).
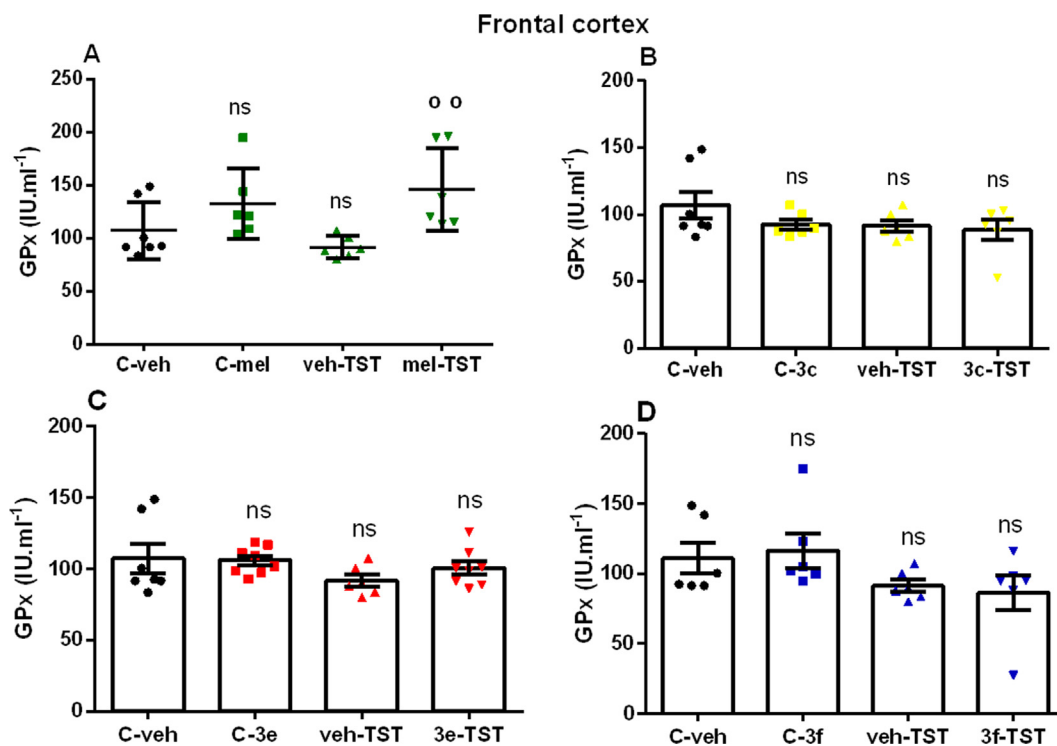
Frontal cortex

Fig. 12. Effects of melatonin (A) and the compound **3c** (B), **3e** (C), and **3f** (D) on GPx activity (ng/ml) measured in the FC in naïve mice (C-mel, C-**3c**, C-**3e** and C-**3f**) and stressed by TST mice (mel-TST, **3c**-TST, **3e**-TST and **3f**-TST) (n = 6). Details as in Fig. 10. ^{oo}P = 0.007 TST-mel compared to veh-TST (A).
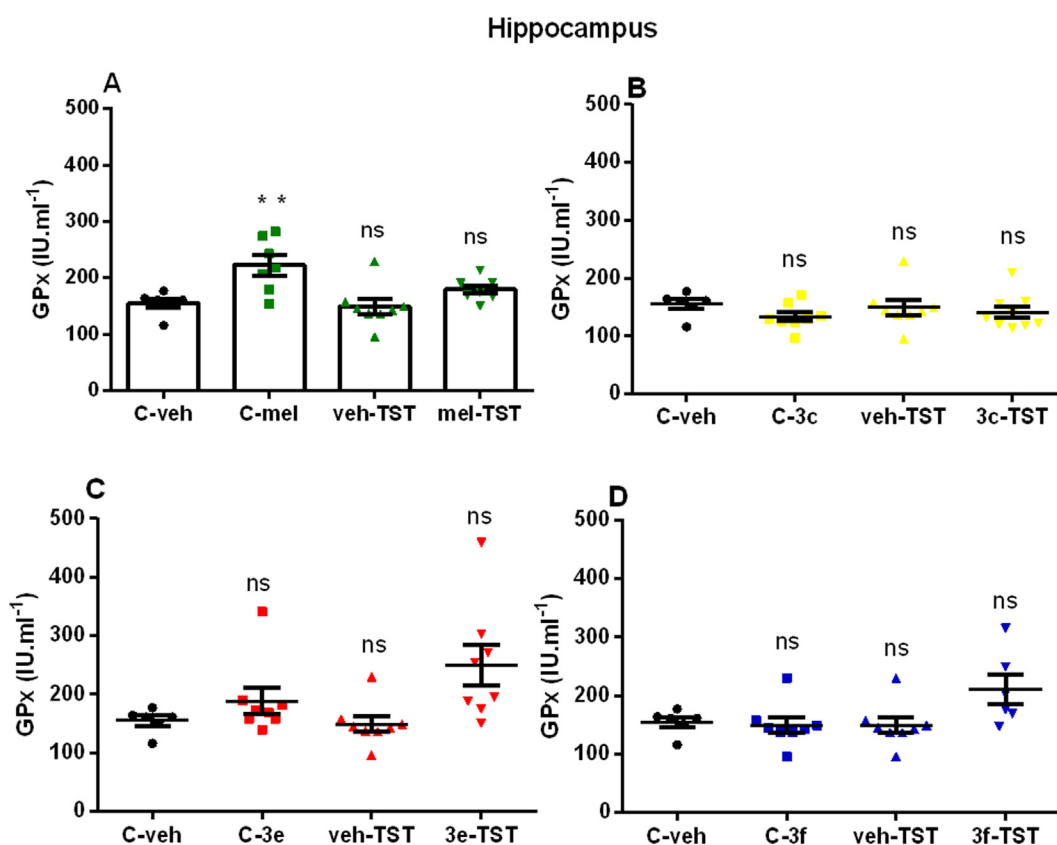
Hippocampus

Fig. 13. Effects of melatonin (A) and the compound **3c** (B), **3e** (C), and **3f** (D) on GPx activity (ng/ml) measured in the hippocampus in naïve mice (C-mel, C-**3c**, C-**3e** and C-**3f**) and stressed by tail suspension test (TST) mice (mel-TST, **3c**-TST, **3e**-TST and **3f**-TST) (n = 6). Details as in Fig. 10. **P = 0.0081C-mel compared to veh-C group (A).
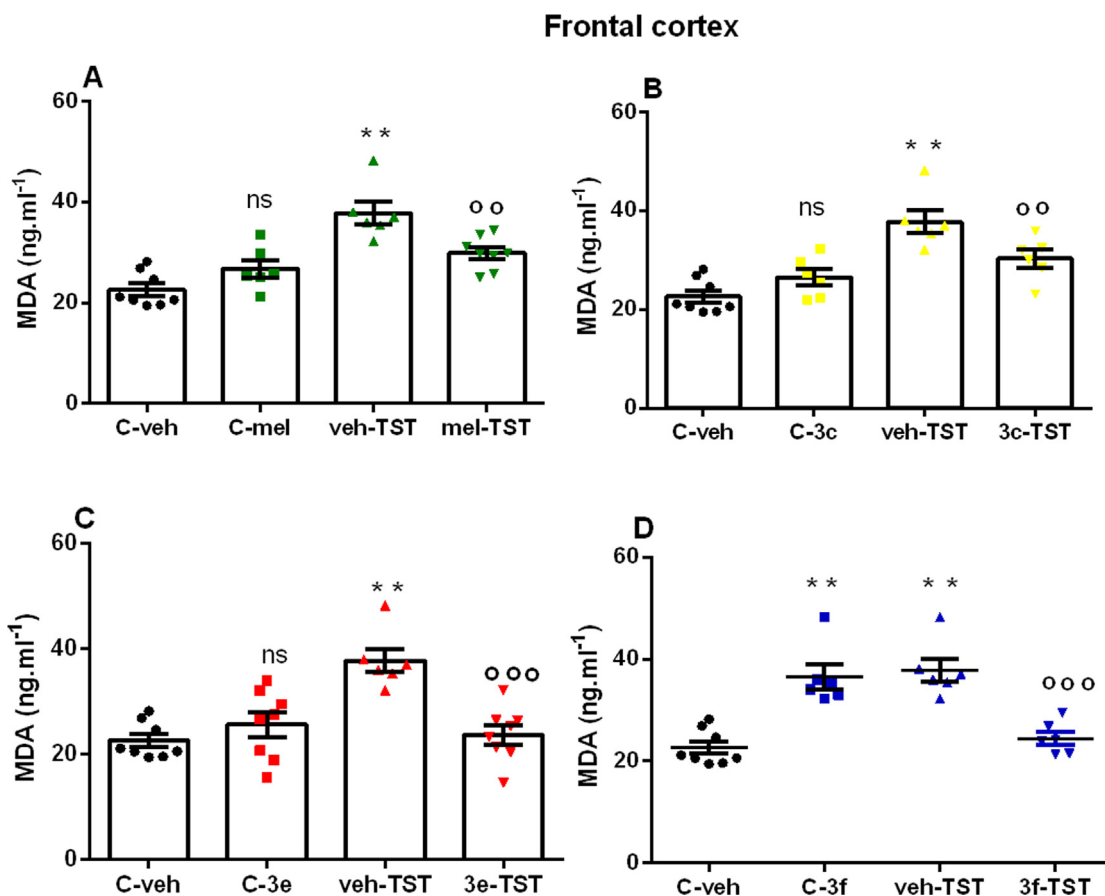
Frontal cortex

Fig. 14. Effects of melatonin (A) and the compound **3c** (B), **3e** (C), and **3f** (D) on the level of MDA levels (ng/ml) measured in the FC in naïve mice (C-mel, C-**3c**, C-**3e** and C-**3f**) and stressed by tail suspension test (TST) mice (mel-TST, **3c**-TST, **3e**-TST and **3f**-TST) (n = 6). Details as in Fig. 10. **P = 0.01 veh-TST compared to veh-C group (A), ^{oo}P = 0.0085 mel-TST compared to veh-TST group (A); ^{oo}P = 0.007 **3c**-TST compared to veh-TST (B), ^{ooo}P = 0.001 **3e**-C compared to veh-C group (C), ^{ooo}P = 0.001 **3f**-TST compared to veh-TST (D).

inflammation (Ray et al., 2004). The analgesic effect of melatonin has been shown to involve MT1 and MT2 receptors present in the spinal cord and various brain regions. The contribution of opioid receptors (Arreola-Espino et al., 2007), as well as by gamma-aminobutyric acid (GABAergic) systems (Wu et al., 2000), alpha-1 adrenergic and 5HT$_2$ and 5HT$_3$ serotoninergic receptors are also proposed (Ray et al., 2004). In our previous work, we have reported the docking results of **3c, 3e,** and **3f** for the MT1 receptor. So, we do expect that melatonin analogs also will display analgesic activity. Moreover, it is already known by the other investigators that substitution of a heterocyclic moiety at 3-position of the indole ring is important for the anti-inflammatory activity (Verma et al., 1994). We also demonstrated that the 2-chlorophenyl substitution is important for the analgesic activity of the **3c** compound, providing analgesia at a lower dose of 60 mg.kg compared melatonin (100 mg.kg) in the first phase of the formalin test. Additional experiments are planned for the assessment of possible anti-inflammatory effects of the tested substances in the near future.

4.3. Antioxidant effect of the tested substances

In *Experiment#3,* we have evaluated the activity of two of the most important antioxidant enzymes, SOD and GPx, and the lipide peroxidation as MDA after administration of relatively high doses of melatonin or melatonin analogs in TST-induced oxidative stress. Similarly to melatonin, the three melatonin analogs (**3c**, **3e**, and **3f**) decreased lipid peroxidation in the FC, but not in the hippocampus. SOD and GPx activities were increased both in the FC and the hip-

pocampus after the application of melatonin. This effect is in line with the observation made by other investigators (Goc et al., 2017). An increase of SOD-activity only in the FC was observed after applying **3c** and **3f**, but not **3e**. In contrast to the melatonin, the three melatonin derivatives failed to increase the hippocampus's antioxidant enzyme activity.

Several mechanisms can be hypothesized, i.e., melatonin's direct action on the antioxidant enzymes, signal transduction, and gene expression regulation. Both melatonin and its metabolites change the cellular redox state by activating transcription regulators of antioxidant enzymes, such as AP-1 and NF-kB. (Moniruzzaman et al., 2018).

The direct free radical scavenging properties by the melatonin molecule were widely studied, and it was concluded that this could only happen with supraphysiological concentrations (Matuszak et al., 1997). The melatonin's indole ring serves as a reactive antioxidant center with its high resonance stability and low activation energy. However, the methoxy and amide side chains also contribute significantly to melatonin's antioxidant capacity (Tan et al., 2002). The methoxy group in the 5-position of the melatonin analogs bearing hydrazine/hydrazone moiety did not affect the *in vitro* antioxidant activity (Suzen et al., 2012). On the other hand, N-methylindole derivatives bearing hydrazine/hydrazone moiety protected against membrane lipid peroxidation and decreased hemolysis of human erythrocytes (Shirinzadeh et al., 2010), and some indole-3-aldehyde hydrazones demonstrated antioxidant and neuroprotective potential in a cell-cultures study (Gurer-Orhan et al., 2016).
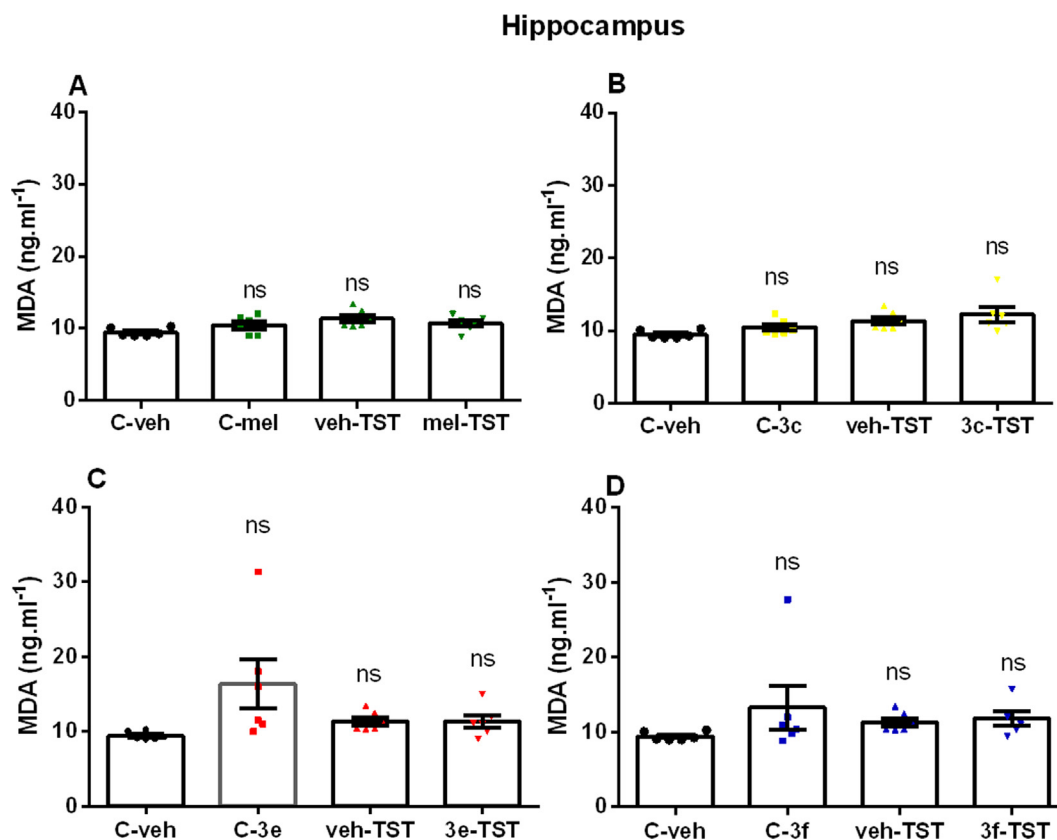
Hippocampus

Fig. 15. Effects of melatonin (A) and the compound **3c** (B), **3e** (C), and **3f** (D) on the level of MDA levels (ng/ml) measured in the hippocampus in naïve mice (C-mel, C-**3c**, C-**3e** and C-**3f**) and stressed by tail suspension test (TST) mice (mel-TST, **3c**-TST, **3e**-TST and **3f**-TST). Two-way ANOVA was applied. Other details are as in Fig. 10.

The methoxy group is preserved, and the amide side chain is substituted by either benzohydrazide or carbohydrazide moieties in all three tested by us melatonin analogs. The decreased lipid peroxidation subsided. Our *in vivo* experiments are entirely in line with the *in vitro* experiments, reported by other investigators, that melatonin analogs with substituted hydrazone side-chain in the 3-position posses a substantial antioxidant activity (Gurkog et al., 2009). In the body, hydrazones metabolize via NAD + -dependent oxidation reaction and may gain or increase a carbonyl-scavenging activity. This peculiarity makes the melatonin analogs' advantages over melatonin (Suzen et al., 2012).

We sought to confirm that compounds with p-Cl on the phenyl ring have significantly higher antioxidant activity (Gürkök et al., 2009), as is the case with the **3c** substance from our experiments.

The melatonin is devoid of prooxidant activity in the opposite of the other antioxidant molecules (Galano et al., 2011). However, with the **3f** melatonin analog's thienyl substitution, it seems that prooxidant activity was added, leading to an increase of the MDA in the FC. Our previous work has already demonstrated an increased lipid peroxidation of the substance 3f for rat hepatocytes (Angelova et al., 2019).

4.4. Conclusion

In the present study, the three melatonin analogs bearing hydrazide/hydrazone substitution at 3C of the indol scaffold demonstrated improved antidepressant-like activity compared to the melatonin. The tested substances are devoided of anxiolytic effects. The antioxidant activity of the melatonin analogs and analgesic potential is comparable to that of melatonin. Based on our docking study (Tchekalarova et al., 2019) performed with the three melatonin analogs **3c**, **3e**, and **3f**, we propose that the antidepres-sant and analgesic effects are probably involving MT1 receptors. However, other mechanisms, i.e., MAO inhibition anti-inflammatory, mediated by different receptors, merit further evaluations. The 3C substitution with hydrazide/hydrazone moiety substantially contributes to the antidepressant and antioxidant activity of the melatonin analogs.

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