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ORIGINAL INVESTIGATION

Psychoactive properties of BNN27, a novel neurosteroid derivate, in male and female rats

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Abstract

Rationale Neurosteroids, like dehydroepiandrosterone (DHEA), play an important role in neurodegeneration and neural protection, but they are metabolized in androgens, estrogens, or other active metabolites. A newly developed synthetic DHEA analog, BNN27 ((20*R*)-3β,21-dihydroxy-17*R*,20-epoxy-5-pregnene), exerts neurotrophic and neuroprotective actions without estrogenic or androgenic effects.

Objectives This study aimed to investigate potential anxiolytic or antidepressant properties of BNN27.

Methods Male and female adult Wistar rats were treated with BNN27 (10, 30, or 90 mg/kg, i.p.) and subjected to behavioral tests measuring locomotion, exploration, and "depressive-like" behavior (open field, light/dark box, hole-board, and forced swim tests). The hippocampus and prefrontal cortex were collected for glutamate and GABA measurements, and trunk blood was collected for gonadal hormone analysis.

Results Acute high-dose BNN27 reduced locomotion and exploratory behavior in both sexes. Intermediate acute doses (30 mg/kg) of BNN27 reduced exploration and testosterone levels only in males, and enhanced progesterone levels in both sexes. Notably, with the present design, BNN27 had neither anxiolytic nor antidepressant effects and did not affect estrogen levels. Interestingly, acute administration of a low BNN27 dose (10 mg/kg) increased glutamate turnover, GABA, and glutamine levels in the hippocampus. The same dose also enhanced glutamate levels in the prefrontal cortex of males only. Sex differences were apparent in the basal levels of behavioral, hormonal, and neurochemical parameters, as expected.

Conclusions BNN27 affects locomotion, progesterone, and testosterone levels, as well as the glutamatergic and GABAergic systems of the hippocampus and prefrontal cortex in a sex-dependent way.

Keywords GABA · Steroids · Neuroactive steroids · Sex differences · Glutamate · Behavior · Hippocampus

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Introduction

Neuroactive steroids, which are produced in peripheral tissues, and neurosteroids, synthesized in the central nervous system (Cai et al. 2018), are key endogenous molecules that are involved in a variety of functions, including protection against neuronal apoptosis, neurodegeneration, and promotion of neurogenesis (Charalampopoulos et al. 2008; Reddy and Estes 2016). Decreased neurosteroid synthesis is associated with normal ageing, as well as neurologic disorders, such as Alzheimer's disease and multiple sclerosis (Charalampopoulos et al. 2008; Powrie and Smith 2018). Additionally, neurosteroids have been implicated in the pathophysiology of cognitive deficits associated with neuropsychiatric disorders, such as schizophrenia, depression, and anxiety disorders (Farb and Ratner 2014; van Wingen et al. 2007).

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S) are two of the most abundant neuroactive steroids. They are synthesized in the human adrenal cortex as well as the brain, by both neurons and glia (Agis-Balboa et al. 2006; Calogeropoulou et al. 2009; Mensah-Nyagan et al. 1999). DHEA is known to increase the effects of the excitatory neurotransmitter glutamate (Bergeron et al. 1996), decrease the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Majewska 1992), and stimulate acetylcholine (Ach) release in the hippocampus (Rhodes et al. 1996). Interestingly, all of these neurotransmitters have altered levels in patients with depression and stress-related disorders, suggesting the potential use of neurosteroids as a therapeutic tool (Zorumski et al. 2013). DHEA exerts its neurotrophic actions by binding to the tyrosine kinase (Trk) and pan-neurotrophin p75 ($p75^{NTR}$) receptors of nerve growth factor (NGF), activating these receptors and leading to neuroprotective, pro-survival, and neurogenic effects (Charalampopoulos et al. 2008; Charalampopoulos et al. 2004; Dechant and Barde 2002; Lazaridis et al. 2011; Pediaditakis et al. 2016a; Pediaditakis et al. 2015; Pediaditakis et al. 2016b). It should be noted that p75NTR signaling is involved in both cell survival and death (Dechant and Barde 2002). DHEA sulfate (DHEA-S) is also an important neuroactive steroid and shares many of the antiapoptotic and protective effects of DHEA (Charalampopoulos et al. 2008; Charalampopoulos et al. 2004). Notably, neurotrophins including NGF and BDNF have also been implicated in depression and anxiety disorders, and also exhibit significant differences between male and female patients, highlighting the importance of gender differences in neuropsychiatric disorders (de Azevedo et al. 2014; Kokras and Dalla 2017; Mondal and Fatima 2019).

Despite the neuroprotective role of DHEA in the CNS, its potential clinical use is hampered by its side effect profile: as it is metabolized into estrogens, androgens, or progestins, it exerts endocrine effects, including possibly (but not definitely) hormone-dependent neoplasias (Klinge et al. 2018; Webb et al. 2006). To overcome these drawbacks, several DHEA derivatives with modifications at positions C3 and C17 of the steroid skeleton have been synthesized, with compound BNN27 ((20*R*)-3*β*,21-dihydroxy-17*R*,20-epoxy-5-pregnene) being the most potent (Fig. 1) (Calogeropoulou et al. 2009). BNN27 readily crosses the blood-brain barrier, with very minor back-pumping via transmembrane proteins; it enters the mouse brain within 30 min and in vitro studies showed that it is metabolized by hepatocytes from both mice (half-life range: 9.2-104 min, mean: 56 min) and humans (half-life, 123.1 min) (Bennett Jr. et al. 2016). Unpublished studies by authors IC and AG showed that the estimated half-life of BNN27 in rats is 64 min. BNN27 exerts antiapoptotic and neuroprotective activity through the selective activation of TrkA and $p75^{NTR}$ receptors, like its parent molecule, but it is devoid of the androgenic or estrogenic activity (Calogeropoulou et al. 2009; Pediaditakis et al. 2016a). BNN27 has shown neuroprotective effects in animal models of ALS (Glajch et al. 2016), demyelination (Bonetto et al. 2017), retinal detachment (Tsoka et al. 2018), ketamine-induced psychosis (Zoupa et al. 2019), scopolamine-induced cognitive deficits (Pitsikas and Gravanis 2017), and a model of retina degeneration in the streptozotocin-induced diabetic rats (Iban-Arias et al. 2018).

Although BNN27's neurotrophic and neuroprotective actions have been recently studied, its potential psychoactive properties, specifically relating to anxiety and depression, have not been investigated to date. Therefore, the purpose of the present study was to screen and evaluate BNN27 for potential anxiolytic and antidepressant actions on both male and female rats, as well as to identify possible associated changes in glutamate and γ-aminobutyric acid (GABA) neurotransmission.

Materials and methods

Animals and treatments

In this study, we used 3-month-old male and female, outbred, non-husbanded, normally cycling Wistar rats (estrous cycle length 4–5 days), obtained from the Hellenic Pasteur

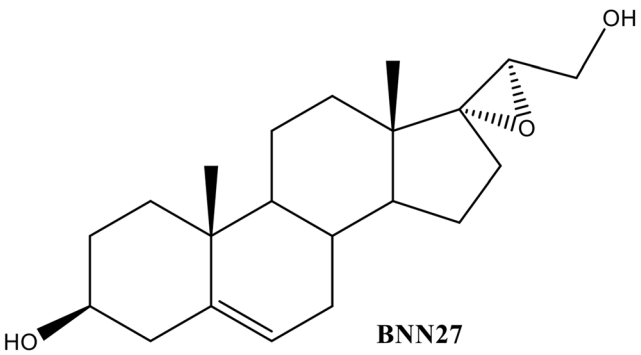


Fig. 1 Structure of BNN27 ((20R)-3β,21-dihydroxy-17R,20-epoxy-5-pregnene)

Institute (Athens, Greece), group-housed according to sex (n = 3–4 per cage), in the same room, under standard animal housing conditions (lights on from 8 a.m. to 8 p.m.; room temperature 22 °C; relative humidity 55%, ad libitum access to food and water) living in plexiglass cages (480 × 375 × 210 mm). All rats were handled in accordance with the guidelines for the care and handling of laboratory animals in EU Directive 2010/63 and the experiments were approved by the local committee (Prefecture of Athens, Greece, protocol number 3288/15 May 2012). The rats were sequentially numbered. Their identification numbers were entered into a spreadsheet software and randomly assigned to treatment groups. Animals remained in each treatment group for the duration of the experiment, and where repeatedly used for behavioral tests and, finally, brain tissue analysis, following administration of their assigned dosage, as described below. Female rats were not tested in a specific stage of the cycle. For the open field, all rats received intraperitoneally (i.p.) one of three doses of BNN27 (10, 30, 90 mg/kg) or vehicle (dimethyl sulfoxide 1 ml/kg). All treatments were volume and weight corrected and all animals received the same dose of vehicle. The vehicle injection volume used herein (1 ml/kg) corresponds to 10.1% of the LD50 of dimethyl sulfoxide, well below the recommendation to not exceed 25% of the LD50 (LD50 = 9.9 ml/kg (Bartsch et al. 1976)), whereas the dose administered in other experiments found in the literature has been as high as 6 ml/kg (Nadruz Jr. et al. 2004). No toxicity effects were observed due to this vehicle exposure, nor any animal suffering, in accordance with previous observations (Castro et al. 1995; Noel et al. 1975; Worthley and Schott 1969) and previous studies involving water insoluble hormone and hormone-like treatments (Ansonoff et al. 2006; Atkins et al. 1998; Gibson and Murphy 2004; Kumon et al. 2000; Tronson et al. 2008; Wong et al. 2012). However, rats receiving the highest BNN dose of 90 mg/kg showed signs of locomotor depression (see also results of the open field) and recovered slowly. This effect cannot be attributed to the vehicle, as all rats received the same weight and volume-corrected amount, but rather to an effect of the high BNN dose. Therefore, no further tests were performed with this dose. All acute treatments were administered 30 min prior to each behavioral test or the tissue sampling, based on the previous finding that BNN27 and its metabolites are detected in the mouse brain 30 min after i.p. injection in mice (Bennett Jr. et al. 2016). This timepoint is in accordance with acute anxiolytic treatments in rodent behavioral testing (Crawley 1981). A different, standard dosage scheme was followed in the forced swim test as described below. An overview of the experimental design is available in Fig. 2.

Behavioral testing

Open field test

Locomotor activity was assessed in an Open Field apparatus (square arena (43 cm × 43 cm × 30 cm)) surrounded by tall Plexiglas walls (Med Associates Inc., St. Albans City, VT, USA). All rats were exposed to the open field after being acclimated to the test room for 1 h prior to testing (Total n = 71; vehicle: males n = 11, females n = 10; BNN27 10 mg/kg: males n = 10, females = 9; BNN27 30 mg/kg: males n = 10, females n = 10, BNN27 90 mg/kg: males n = 6, females n = 5). As explained above, rats receiving the highest BNN dose of 90 mg/kg showed acute signs of locomotor suppression, so treatment administration was stopped at 6 males and 5 females. Rats were placed in the center of the arena and allowed to explore the area for 10 min. At the end of the trial, all animals were returned to their home cage and the arena was cleaned with ethanol 70%. Horizontal and vertical activity were measured using the Activity Monitor software version 5 (Med Associates, Fairfax, VT, USA), as described previously (Kafetzopoulos et al. 2018; Kokras et al. 2018).

Light/Dark box test

In order to assess locomotion and anxiety, rats were placed in the illuminated compartment of a Light/Dark (L/D) box for 10 min. For this test, in order to confirm the behavioral response, an additional group of rats having received a single BNN27

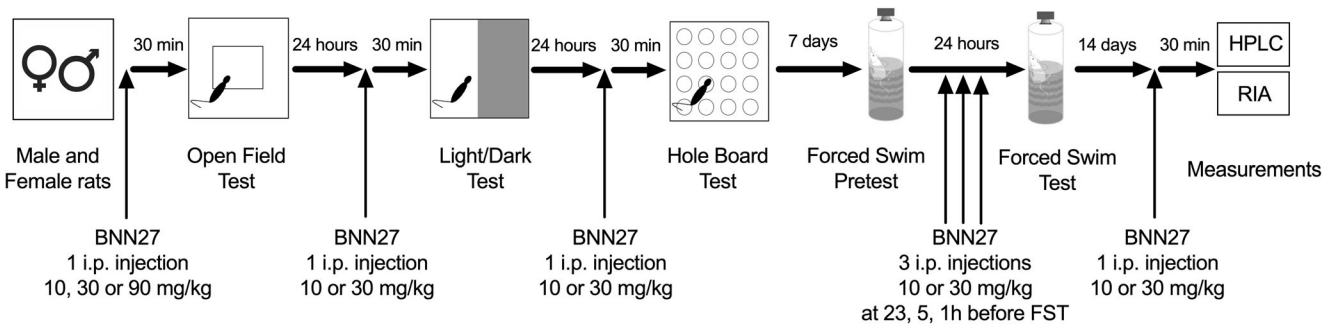


Fig. 2 Timeline of the experiment and graphical depiction of the experimental procedures. Vertical arrows indicate the BNN27 intraperitoneal (i.p.) administration. HPLC (High-Performance Liquid Chromatography) refers to the neurochemical assay of glutamate, GABA, and glutamine. RIA (Radioimmunoassay) refers to the measurement of testosterone, estradiol, and progesterone. For details, see the methods section

injection 30 min prior to the test was used only for the L/D test, along with all rats that were subjected to the behavioral battery. Results are presented pooled as there were no statistical differences between the two batches. All rats (total n = 94; all groups n = 16, except males BNN27 10 mg/kg: n = 15 and males BNN27 30 mg/kg: n = 15) were exposed to the L/D after being acclimated to the test room for 1 h prior to testing. The L/D box consisted of a square arena (43 cm × 43 cm × 30 cm) surrounded by tall Plexiglas walls and was divided in two arenas, one illuminated and one dark, sharing the same dimensions (Med Associates Inc., St. Albans City, VT, USA). All trials were video recorded and were manually scored by an observer blind to the treatment of the animal, as before (Kokras et al. 2012). At the end of the trial, all animals were returned to their home cage and the box was thoroughly cleaned with ethanol 70%. The total number of transitions and the duration of time spent in the illuminated compartment were scored and calculated using Kinoscope, an in-house developed computer program (Kokras et al. 2017).

Hole-board test

The hole-board test also measures anxiety-like behavior (File and Wardill 1975; Takeda et al. 1998). Hole-board consisted of a square arena (43 cm × 43 cm × 30 cm) surrounded by tall Plexiglas walls and a board of 16 holes (4-cm diameter, Med Associates Inc., St. Albans City, VT, USA). All rats (total n = 57; all groups n = 10, except males BNN27 10 mg/kg: n = 9 and males BNN27 30 mg/kg: n = 8) were exposed to the hole-board after being acclimated to the test room for 1 h prior to testing. Rats were allowed to explore the apparatus for 10 min. At the end of the trial, all the animals were returned to their home cage and the arena was thoroughly cleaned with ethanol 70%. Total number of holes explored (head-dip counts) and latency to first hole was monitored by using the Activity Monitor software, (Med Associates Inc.). An index was extracted by calculating the ratio of repetitive hole explorations versus the total number of holes visited (Index = Repeat/Total), as a crude estimation of the working memory in this single-session hole-board test without previous training and with un-baited holes (Karl et al. 2006; Lin et al. 2010; van der Staay et al. 2012).

Forced swim test

For monitoring depression-like behavior, all rats were individually subjected to a modified forced swim test (FST), as previously described (Kokras et al. 2015; Kokras et al. 2014; Slattery and Cryan 2012). Specifically, rats (total n = 60, all groups n = 10) were placed individually inside a transparent plexiglass cylinder (50 cm × 19 cm) and subjected to a 15-min pretest session. At the end of the trial, all animals were returned to their home cage and the cylinder was thoroughly washed with tap water. Thereafter, all rats received three injections of BNN27 or vehicle 23, 5, and 1 h before the next FST test session. One hour after the last injection and 24 h after the pretest session, all rats were subjected to a second 5-min FST session in the same way. The FST test trial was video recorded and immobility, swimming, climbing, and head shake behaviors were scored manually as before (Kokras et al. 2015), by using the in-house developed software Kinoscope (Kokras et al. 2017).

Neurochemical amino acid analysis

Two weeks after the last behavioral test, rats (total n = 57, n = 8–10/group) received an injection of BNN27 or vehicle and 30 min later, they were killed by rapid decapitation (Fig. 2). Brain tissue samples from the hippocampus and prefrontal cortex (PFC) were collected for neurochemical assays using high-performance liquid chromatography (HPLC-ED). The number of samples that were used for each measurement was as follows: vehicle group: males n = 7 (GABA-hippocampus), n = 8 (glutamine-hippocampus and glutamate-hippocampus), n = 9 (prefrontal cortex-all neurotransmitters), females n = 9 (all measurements) BNN27 10 mg/kg group: males n = 9 (GABA and glutamate-prefrontal cortex), n = 10 (all other measurements), females: n = 9 (GABA-hippocampus), n = 10 (all other measurements) BNN27 30 mg/kg group: males n = 9 (GABA and glutamine-hippocampus), n = 10 (all other measurements), females: n = 9 (glutamine-prefrontal cortex), n = 10 (all other measurements). Analytical measurements were performed using a LKB2248 (Pharmacia Sweden) HPLC pump coupled with a BAS LC4C (Bioanalytical Systems, West Lafayette, IN, USA) electrochemical detector and pre-column derivatization, as previously described (Kokras et al. 2009; Melo et al. 2015), with some minor modifications. The working electrode was glassy carbon, the reference electrode was Ag/AgCl, and the column used was Aquasil, 250 mm × 4.6 mm, 5 μm (Thermo Fisher Scientific, MA, USA). The voltage of the working electrode was set at + 800 mV. The mobile phase consisted of an acetonitrile (Chem-Lab, Belgium): 100 mM monosodium-phosphate buffer (4:96) pH 5.6, containing 0.5 mM disodium ethylenediaminetetraacetate dihydrate (Na_2EDTA, AppliChem, Germany). Samples were diluted 5:1 with 0.1 M Borax buffer (Sigma–Aldrich, St. Louis, USA), pH 10.4. o-Phthalaldehyde (Sigma–Aldrich) was subsequently added to the samples and left to react at room temperature for 10 min prior to injection. Quantification of glutamate, glutamine, and GABA was done by comparison of the area under the curve with that of reference external standards using HPLC software (Clarity, Data-Apex, Czech Republic). Moreover, given that glutamine (GLN) is glutamate's (GLU) metabolite (Bak et al. 2006; Hertz 2013; Patel et al. 2005) (Fig. 3), we further calculated the GLN/GLU ratio, which corresponds to the glutamate turnover rate, as usually calculated with dopamine and serotonin neurotransmitters and

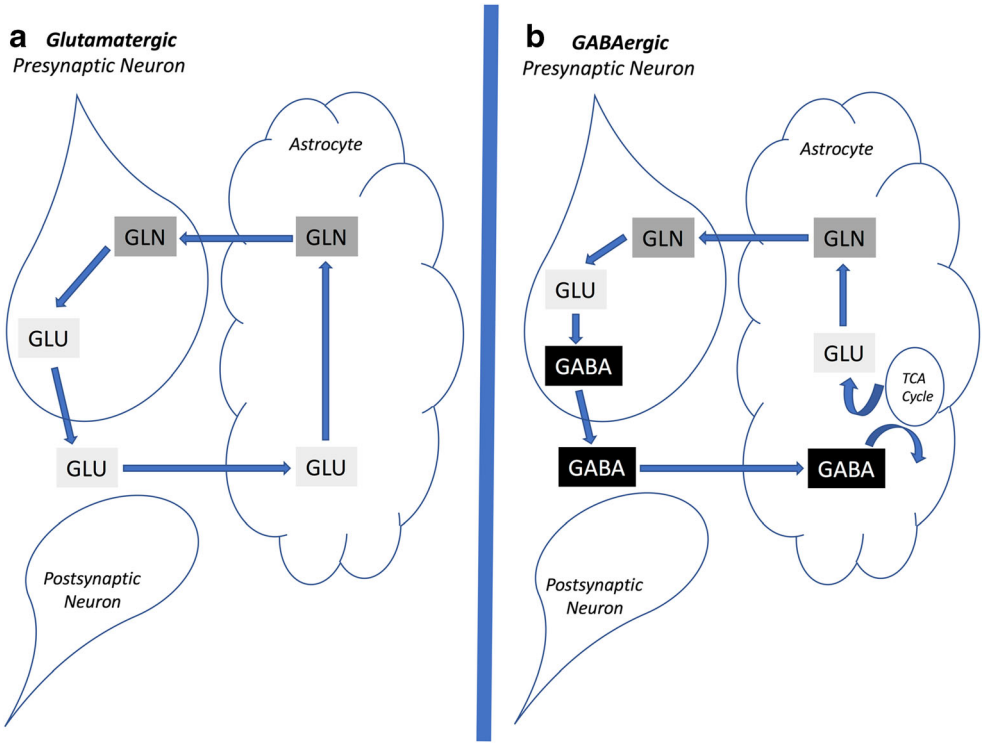


Fig. 3 Glutamate-GABA-glutamine cycle. Simplified graphical depiction of the glutamate (GLU)-GABA-glutamine (GLN) cycle in glutamatergic (**a**) and GABAergic (**b**) neurons. TCA Cycle refers to the tricarboxylic cycle or Krebs cycle

their metabolites (Gemmel et al. 2017; Gemmel et al. 2018; Kokras et al. 2019; Morgado et al. 2015).

Hormone measurements

Trunk blood was collected during the decapitation and processed for serum extraction, as previously described (Kokras et al. 2018; Kokras et al. 2014), and was stored in – 80 °C until analysis. Estradiol, progesterone, and testosterone assays were performed using commercially available Radioimmuniassay (RIA) kits (Estradiol Double Antibody, Siemens Healthineers, Erlangen, Germany; Progesterone Coat-A-Count, Siemens Healthineers, Erlangen, Germany; Testosterone Double Antibody, MP Biomedicals, Santa Ana, CA, USA), as previously (Kokras et al. 2015). The detection limits were 4 ng/dl, 0.02 ng/ml, and 1.4 pg/ml respectively. The total number of samples is $n = 46$, and the number breakdown for each measurement is as follows: vehicle group: males $n = 7$, females $n = 7$ (estrogen and progesterone) or $n = 5$ (testosterone); BNN27 10 mg/kg group: males: $n = 8$, females $n = 8$ (estrogen and progesterone) or $n = 7$ (testosterone); BNN27 30 mg/kg group: males $n = 8$, females $n = 8$.

Statistical analysis

Results were analyzed with SPSS v.25 (IBM Corp, NY, USA). For all experiments, a two-way analysis of variance (ANOVA) was performed with Sex (male; female) and Treatment (VEH, BNN27) as independent variables. Data were checked for homogeneity of variance and if necessary, they were cubic root transformed to meet ANOVA requirements. Treatment main effects were further analyzed using Dunnett's post hoc test comparing different BNN27 doses against the vehicle treatment group. Significant Sex × Treatment interactions were further explored with post hoc pairwise comparisons. For all factorial comparisons, estimates of effect size are provided in the form of partial eta squared (η^2). Values of $p \leq 0.05$ were considered statistically significant. Numerical data is presented as means ± standard error of the mean (SEM). The graphs were created with GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

Results

Behavioral results

Open field test

A two-way ANOVA for the distance travelled in the open field revealed significant Sex and Treatment main effects ($F_{(1,63)} = 5.595$, $p = 0.021$, $\eta^2 = 0.082$; $F_{(3,63)} = 7.280$, $p < 0.001$, $\eta^2 = 0.257$ respectively). Female rats travelled a longer distance than males, and post hoc testing showed that the highest 90 mg/kg BNN27 dose resulted in a marked decrease in horizontal locomotion in both sexes ($p < 0.001$) (Fig. 4a), whereas the 10 mg/kg and 30 mg/kg BNN27 doses were not statistically different from vehicle treatment. The two-way

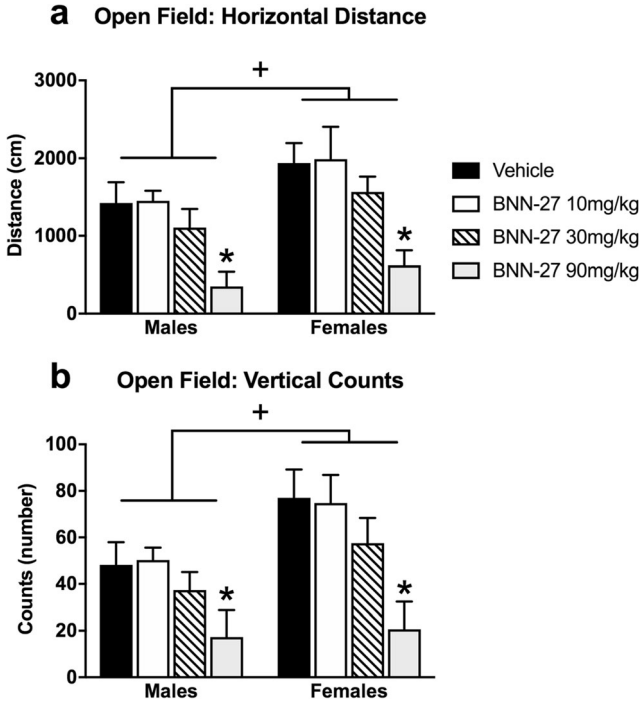


Fig. 4 Horizontal (**a**) and vertical (**b**) activity of male and female rats during a 10-min open field test. Male and female rats received an i.p. injection of vehicle (males $n = 11$, females $n = 10$), BNN27 10 mg/kg (males $n = 10$, females $n = 9$), BNN27 30 mg/kg (males $n = 10$, females $n = 10$), or BNN27 90 mg/kg (males $n = 6$, females $n = 5$), 30 min before being placed to the open field apparatus. Graphs depict means ± SEM. The cross (+) denotes a Sex main effect, as females travelled a longer horizontal distance ($p = 0.021$) and exhibited more vertical counts ($p = 0.010$), in comparison with males. The asterisk (*) denotes a post hoc Treatment effect, as only the highest BNN27 dose (90 mg/kg) significantly reduced the horizontal distance ($p < 0.001$) and vertical counts ($p < 0.001$) in both sexes

ANOVA for the rearing behavior during the open field (as measured by the vertical counts) showed significant Sex and Treatment main effects ($F_{(1,63)} = 7.056$, $p = 0.010$, $\eta^2 = 0.101$; $F_{(3,63)} = 6.497$, $p < 0.001$, $\eta^2 = 0.236$ respectively). Female rats displayed more vertical counts, but post hoc testing showed that the BNN27 90 mg/kg dose significantly and markedly reduced vertical counts in both sexes ($p = 0.001$), whereas the 10 mg/kg and 30 mg/kg BNN27 doses did not (Fig. 4b). Given the considerable suppression of motor activity following the highest BNN27 90 mg/kg dose and the general malaise of the animals, this dose was not further used in other behavioral tests.

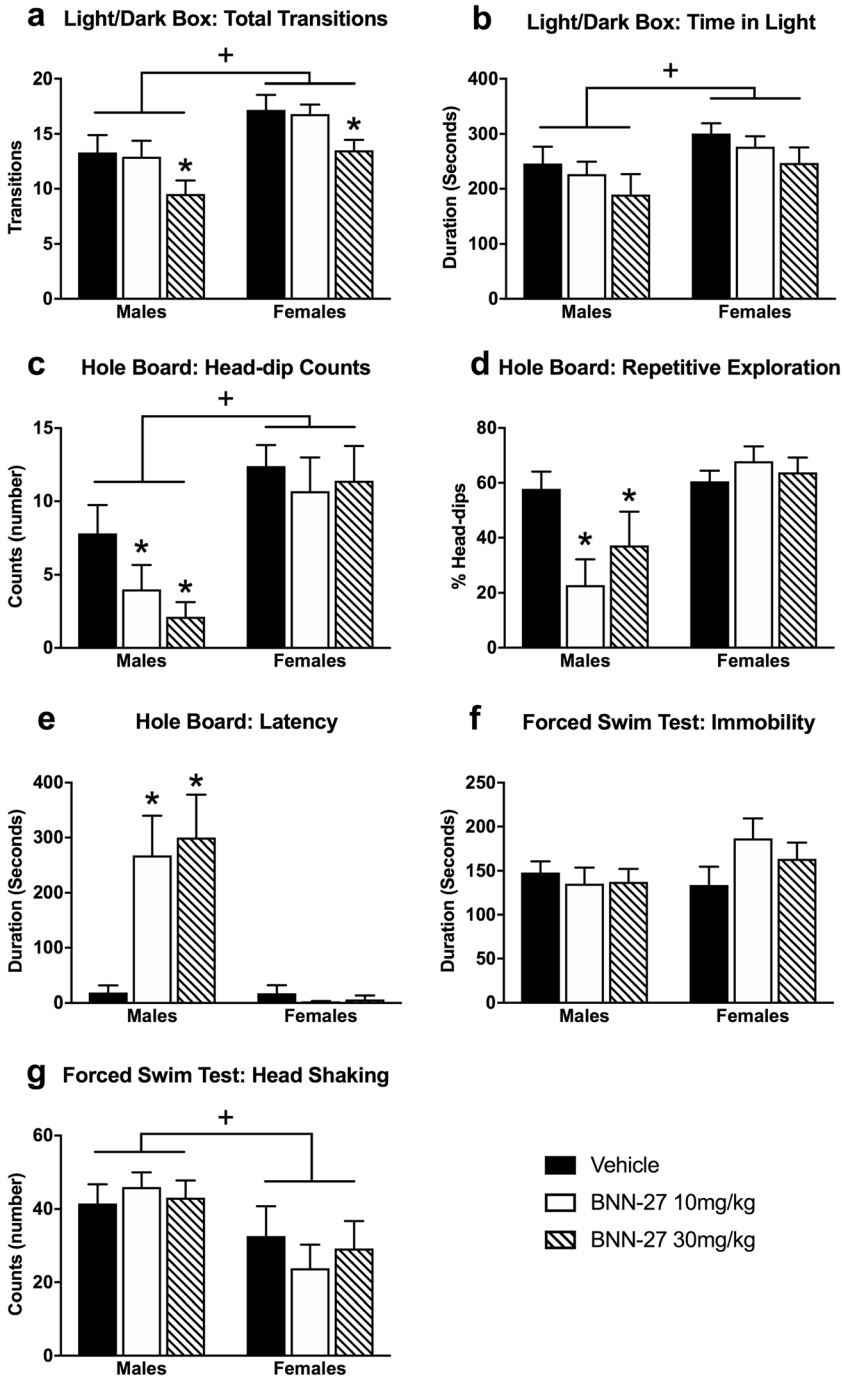
Light/dark box

A two-way ANOVA for the number of total transitions between the light and the dark compartment of the box showed a significant Sex main effect ($F_{(1,88)} = 14.316$, $p < 0.001$, $\eta^2 = 0.140$) as females displayed more transitions between the two compartments and a Treatment main effect ($F_{(1,88)} = 5.264$, $p = 0.007$, $\eta^2 = 0.107$). Post hoc testing showed that the 30 mg/kg BNN27 dose reduced the number of transitions ($p = 0.009$) (Fig. 5a). A two-way ANOVA for the time spent in the light compartment showed a significant Sex main effect ($F_{(1,88)} = 6.103$, $p < 0.015$, $\eta^2 = 0.065$) as females spent more time in the light compartment than males, but the same analysis did not show any statistically significant BNN27 effects on time spent in the light compartment (Fig. 5b).

Hole-board test

A two-way ANOVA for the total number of explored holes (head dips) during the hole-board test showed a significant Sex × Treatment interaction ($F_{(2,51)} = 3.716$, $p = 0.031$, $\eta^2 = 0.127$) and post hoc testing showed that both BNN27 treatments (10 mg/kg and 30 mg/kg) reduced the total number of holes explored by the male animals ($p = 0.002$; $p = 0.002$ respectively) (Fig. 5c). Regarding the total number of holes explored, no BNN27 effects were seen in female rats. However, female rats explored more holes than males ($p < 0.001$ for all group comparisons). A two-way ANOVA for the

Fig. 5 Effects of sex and BNN27 treatment in the light/dark box, hole-board, and forced swim tests. Male and female rats received an i.p. injection of vehicle, BNN27 10 mg/kg, or BNN27 30 mg/kg 30 min before being subjected to the light/dark box (total $n = 94$; all groups $n = 16$, except males BNN27 10 mg/kg: $n = 15$ and males BNN27 30 mg/kg: $n = 15$) and hole-board tests (total $n = 57$; all groups $n = 10$, except males BNN27 10 mg/kg: $n = 9$ and males BNN27 30 mg/kg: $n = 8$), and three injections of the same dosages, 23 h, 5 h, and 1 h before the forced swim test session (total $n = 60$, all groups $n = 10$). Graphs depict means ± SEM. Light/dark box test-total transitions (**a**): The cross (+) denotes a Sex main effect in the total transitions between the dark and the light compartments, as females displayed higher counts ($p < 0.001$). There was also a Treatment effect (post hoc effects indicated by an asterisk (*)), as BNN27 30 mg/kg reduced the number of transitions ($p = 0.009$). Light/dark box test-time in light (**b**): The cross (+) denotes a Sex main effect in the duration the rat spent in the light area initially, before transitioning to the dark area. Females displayed overall higher counts ($p < 0.015$). There was no Treatment effect. Hole-board test-head-dip counts (**c**): The cross (+) denotes a Sex main effect in the total number of head dips inside the holes, as females displayed overall higher counts ($p < 0.001$). Both the 10 mg/kg and 30 mg/kg BNN27 treatments reduced head dips in males only ($p < 0.001$ and $p = 0.009$ respectively, post hoc effects indicated by an asterisk (*), whereas there was no Treatment effect in females. Hole-board test-repetitive exploration (**d**): The asterisks (*) denote a Treatment effect in the percentage of head dips in holes that were explored before. Both the 10 mg/kg and 30 mg/kg BNN27 treatments reduced the repetitive exploration in males only ($p < 0.001$ and $p = 0.002$ respectively). Hole-board test-latency (**e**): The asterisks (*) denote a Treatment effect in the latency to explore the first hole. Both the 10 mg/kg and 30 mg/kg BNN27 treatments increased the latency in males only ($p = 0.003$ and $p = 0.001$ respectively). A post hoc comparison revealed a significant sex differences between male and female BNN-treated rats. Forced swim test-immobility (**f**): There were no Sex or Treatment effects in immobility time. Forced swim test-head shaking (**g**): The cross (+) denotes an Sex main effect in the frequency of head shakes, as females had lower counts ($p = 0.005$) than males. There was no BNN27 effect on the forced swim test



latency time to explore the first hole showed a significant Treatment × Sex interaction ($F_{(2,51)}$ = 4.152, p = 0.021, η^2 = 0.140) and post hoc testing showed that both BNN27 treatments (10 mg/kg and 30 mg/kg) increased the latency time (p = 0.003; p = 0.001 respectively), only in males (Fig. 5e). As a result, male rats treated with 10 mg/kg and 30 mg/kg BNN displayed increased latency (p = 0.002; p = 0.001 respectively), in comparison with their female counterparts, whereas no sex differences were observed at baseline, following vehicle treatment. Indeed, BNN27 had no effect on the latency to explore the first hole in female rats. Similarly, a two-way ANOVA for the hole-board working memory index showed a significant Treatment × Sex interaction ($F_{(2,51)}$ = 5.112, p = 0.009, η^2 = 0.167] and post hoc testing showed that both BNN27 treatments (10 mg/kg and 30 mg/kg) reduced the hole-board working memory index (p < 0.001; p = 0.009 respectively), only in males (Fig. 5d). On the other hand, BNN27 had no effects in females regarding the hole-board index.

Forced swim test

A two-way ANOVA did not show any statistically significant differences following BNN27 treatment for immobility (Fig. 5f), swimming, and climbing duration during the FST test session (data not shown). Regarding head shaking behavior, a significant Sex main effect was revealed ($F_{(1,54)} = 8.719$, $p = 0.005$, $\eta^2 = 0.139$), as females were shaking their heads less than males during the 5-min FST test session, whereas this behavior was not affected by BNN27 treatment (Fig. 5 g).

Hormonal results

Testosterone

A two-way ANOVA for testosterone serum levels showed a significant Sex × Treatment interaction ($F_{(2,37)} = 3.900$, $p = 0.029$, $\eta^2 = 0.174$). Post hoc testing showed that in males, the higher 30 mg/kg BNN27 dose, but not the lower 10 mg/kg dose, diminished testosterone serum levels in males only ($p = 0.007$) (Fig. 6a). As expected, female rats had markedly lower testosterone serum levels than males ($p < 0.001$).

Estrogen

A two-way ANOVA for estrogen serum levels only showed a significant Sex main effect ($F_{(1,40)} = 26.621$, $p < 0.001$, $\eta^2 = 0.400$), because female rats had higher estrogen levels than males, as expected. No effects of BNN27 treatment on estrogen serum levels were revealed (Fig. 6b).

Progesterone

A two-way ANOVA for progesterone serum levels showed significant Sex and Treatment main effects ($F_{(1,40)} = 12.928$, $p < 0.001$, $\eta^2 = 0.244$; $F_{(2,40)} = 4.888$, $p = 0.013$, $\eta^2 = 0.196$ respectively). As expected, female rats had higher progesterone serum levels than males, but BNN27 30 mg/kg treatment significantly elevated progesterone serum levels in both sexes in comparison with vehicle treatment ($p = 0.006$) (Fig. 6c).

Neurochemical results

Glutamate levels

A two-way ANOVA for glutamate tissue levels in the hippocampus revealed a significant Sex main effect, as females have lower levels of glutamate ($F_{(1,51)} = 5.516$, $p = 0.023$, $\eta^2 = 0.098$) (Fig. 7a) than males. However, no effects of BNN27 treatment were evident. The same analysis for glutamate in the PFC showed a marginally significant Sex × Treatment interaction ($F_{(2,51)} = 2.525$, $p = 0.90$, $\eta^2 = 0.90$), a significant Treatment main effect ($F_{(2,51)} = 3.050$, $p = 0.056$, $\eta^2 =$

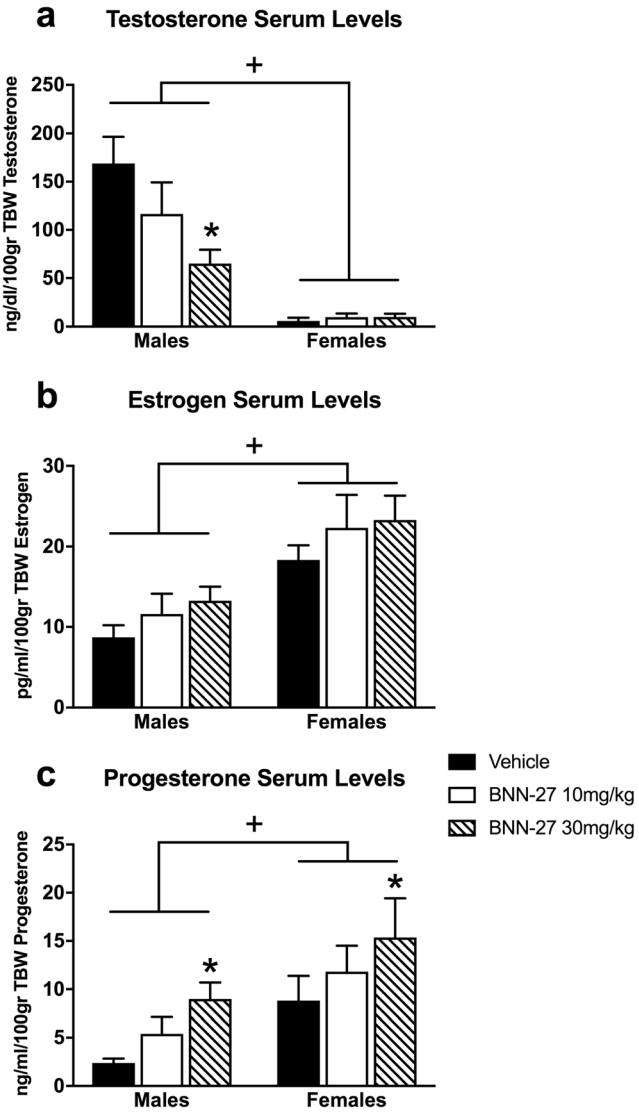


Fig. 6 Effects of sex and BNN27 treatment in serum hormone levels. Two weeks after the last behavioral test, all rats received an i.p. injection of vehicle, BNN27 10 mg/kg, or BNN27 30 mg/kg, and 30 min later, they were killed by rapid decapitation. Testosterone (**a**) was overall significantly lower in females than males ($p < 0.001$), and also BNN27 30 mg/kg decreased testosterone levels in comparison with vehicle ($p = 0.007$). Estrogen (**b**) was overall significantly lower in males than females ($p < 0.001$), without BNN27 Treatment effects. Finally, progesterone (**c**) was overall significantly higher in females than males ($p < 0.001$) and also higher in both male and female rats treated with BNN27 30 mg/kg, in comparison with vehicle ($p = 0.006$). The total number of samples was $n = 46$, and the number of samples used in each measurement is as follows: vehicle group: males $n = 7$, females $n = 7$ (estrogen and progesterone) or $n = 5$ (testosterone); BNN27 10 mg/kg group: males: $n = 8$, females $n = 8$ (estrogen and progesterone) or $n = 7$ (testosterone); BNN27 30 mg/kg group: males $n = 8$, females $n = 8$

0.107), and a significant Sex main effect ($F_{(1,51)} = 7.745$, $p = 0.008$, $\eta^2 = 0.132$), as females had lower glutamate levels in the PFC than males. Post hoc testing also showed that males treated with 10 mg/kg BNN27, but not 30 mg/kg, had higher glutamate tissue levels in their PFC than vehicle-treated males ($p = 0.005$) (Fig. 7b).

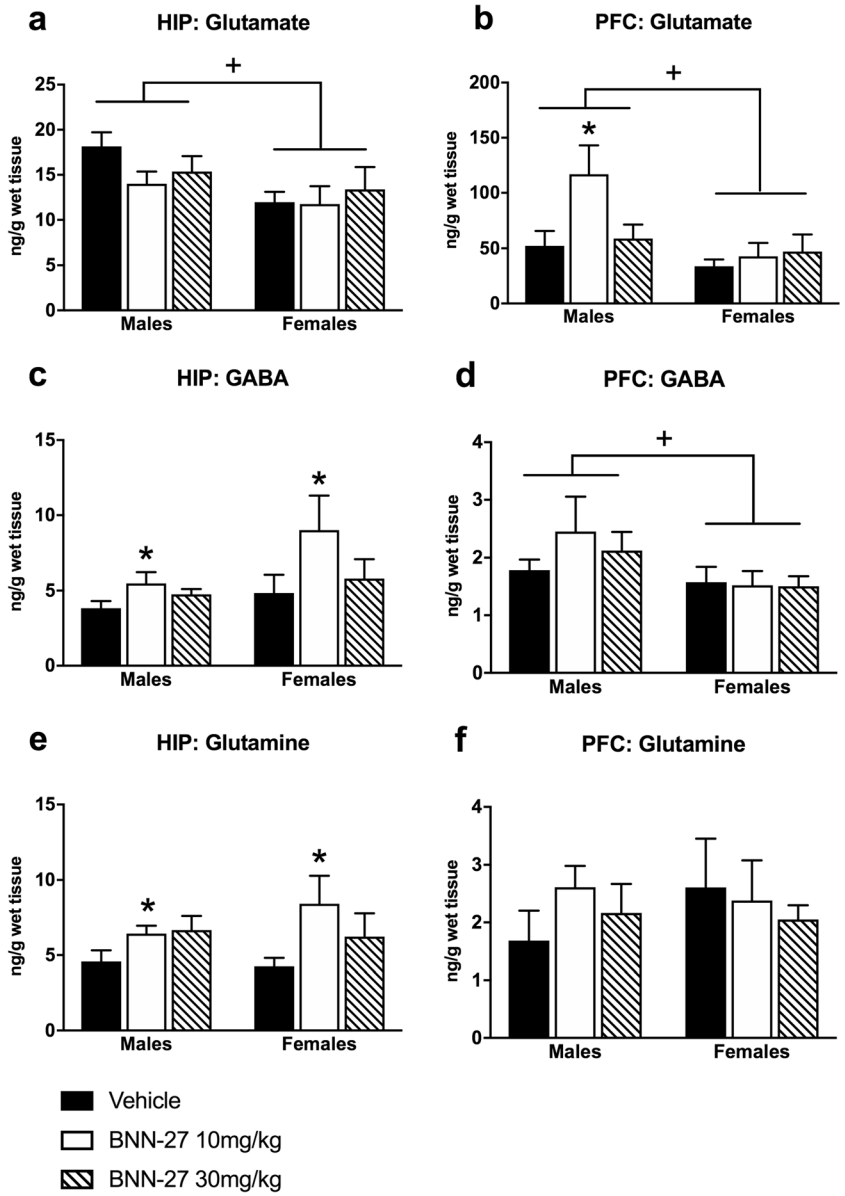


Fig. 7 Effects of sex and BNN27 treatment in brain glutamate, GABA, and glutamine concentrations. Two weeks after the last behavioral test, all rats received an i.p. injection of vehicle, BNN27 10 mg/kg, or BNN27 30 mg/kg, and 30 min later, they were killed by rapid decapitation. In the hippocampus (**a**), glutamate was overall significantly lower in females than males ($^{+}p = 0.023$), whereas there was no Treatment effect. In the prefrontal cortex (**b**), glutamate was significantly lower in females than males ($^{+}p = 0.008$), and significantly higher in males treated with BNN27 10 mg/kg, compared with vehicle ($*p = 0.005$). GABA levels in the hippocampus (**c**) were increased in both male and female rats treated with BNN27 10 mg/kg ($*p = 0.028$). GABA levels in the prefrontal cortex (**d**) were overall lower in females than males ($^{+}p = 0.042$) but were not affected by BNN27 treatment. Hippocampal glutamine (**e**) was significantly increased in male and female rats treated with BNN27 10 mg/kg, compared with vehicle ($*p = 0.019$). There was no Sex or Treatment effect in prefrontal glutamine levels (**f**). The total number of samples was $n = 57$, and the number of samples used in each measurement is as follows: vehicle group: males $n = 7$ (GABA-hippocampus), $n = 8$ (glutamine-hippocampus and glutamate-hippocampus), $n = 9$ (prefrontal cortex-all neurotransmitters), females $n = 9$ (all measurements) BNN27 10 mg/kg group: males $n = 9$ (GABA and glutamate-prefrontal cortex), $n = 10$ (all other measurements), females: $n = 9$ (GABA-hippocampus), $n = 10$ (all other measurements) BNN27 30 mg/kg group: males $n = 9$ (GABA and glutamine-hippocampus), $n = 10$ (all other measurements), females: $n = 9$ (glutamine-prefrontal cortex), $n = 10$ (all other measurements)

GABA levels

A two-way ANOVA for GABA tissue levels in the hippocampus showed a significant Treatment main effect ($F_{(2,48)} = 3.315$, $p = 0.045$, $\eta^2 = 0.121$), and post hoc testing showed the males and female rats treated with 10 mg/kg BNN27 displayed increased GABA levels than vehicle-treated controls ($p = 0.028$) (Fig. 6c). The analysis for GABA levels in the PFC did not reveal any BNN27 Treatment effects but showed a significant Sex main effect ($F_{(1,50)} = 4.341$, $p =$

0.042, η^2 = 0.080), as females had generally lower GABA tissue levels in the PFC than males (Fig. 7d).

Glutamine levels

A two-way ANOVA for glutamine tissue levels in the hippocampus showed a significant Treatment main effect ($F_{(2,50)}$ = 3.626, p = 0.034, η^2 = 0.127). Post hoc testing showed that male and female rats treated with 10 mg/kg BNN27 displayed increased glutamine tissue levels than vehicle-treated controls (p = 0.019) (Fig. 7e). No differences were observed in glutamine tissue levels in the PFC (Fig. 7f).

Glutamate-glutamine cycle

A two-way ANOVA for the glutamine/glutamate ratio (glutamate-glutamine cycle activity) in the hippocampus showed a significant Sex main effect ($F_{(1,50)}$ = 3.824, p = 0.056, η^2 = 0.71), as females had a higher glutamate turnover rate than males, as revealed by the glutamine/glutamate ratio. Moreover, the same analysis showed a significant Treatment main effect ($F_{(2,50)}$ = 6.532, p = 0.003, η^2 = 0.207), and post hoc testing showed that the 10 mg/kg BNN27 dose increased the hippocampal turnover rate in both sexes (p = 0.002), whereas there was a non-significant trend also for the higher 30 mg/kg BNN27 dose (p = 0.072) (Fig. 8a). A similar analysis for the glutamate turnover rate in the PFC showed a significant Sex main effect ($F_{(1,50)}$ = 7.648, p = 0.08, η^2 = 0.133), as females had a higher glutamate turnover rate in comparison with males, but no significant Treatment effects were detected (Fig. 8b).

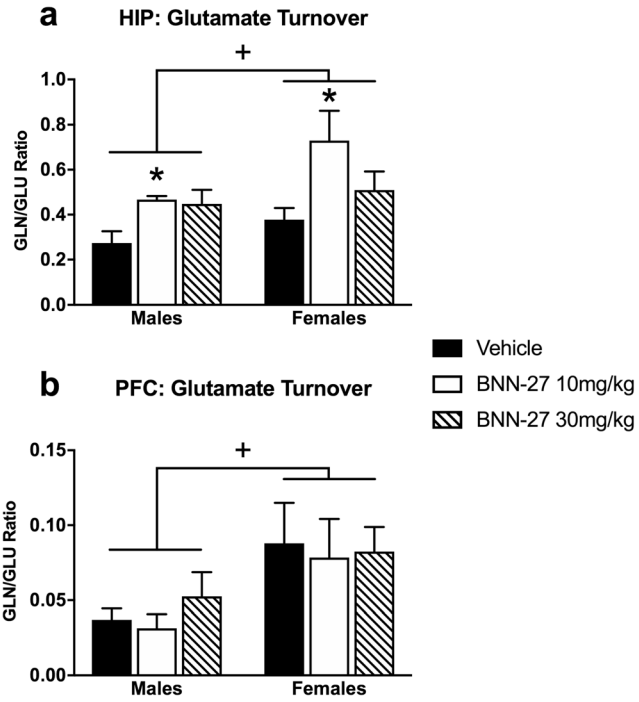


Fig. 8 Effects of sex and BNN27 treatment on glutamate turnover (glutamine/glutamate). Two weeks after the last behavioral test, all rats received an i.p. injection of vehicle, BNN27 10 mg/kg (white bar), or BNN27 30 mg/kg, and 30 min later, they were killed by rapid decapitation. In the hippocampus (**a**), glutamate turnover was overall higher in females than males (^{+}p = 0.056) and higher in both males and females treated with BNN27 10 mg/kg, compared with vehicle ($^{*}p$ = 0.002). In the prefrontal cortex (**b**), glutamate turnover was overall higher in females than males (^{+}p = 0.011), whereas there was no Treatment effect

Discussion

The purpose of this study was to examine the potential antidepressant and anxiolytic effects of BNN27, a synthetic microneurotrophin (termed so due to its small size and its neurotrophic effects), in male and female rats, and identify respective neurochemical changes in the hippocampus and prefrontal cortex. In summary, acute BNN27 administration in very high doses reduced locomotion and exploratory behavior in both sexes. Intermediate acute doses (30 mg/kg) of BNN27 reduced exploration and testosterone levels only in males. The same dose also enhanced progesterone levels in both sexes. Notably, BNN27 had no anxiolytic nor antidepressant effect and did not affect estrogen levels. Interestingly, acute administration of a low BNN27 dose (10 mg/kg) increased glutamate turnover, GABA, and glutamine levels in the hippocampus. The same dose also enhanced glutamate levels in the prefrontal cortex of males only. Overall, BNN27 affects locomotion, progesterone, and testosterone levels, as well as the glutamatergic and GABAergic systems of the hippocampus and prefrontal cortex in a sex-dependent way. Sex differences were also apparent in the basal levels of behavioral, hormonal, and neurochemical parameters, as expected. Specifically, females had higher activity and lower FST head shakes than males, as well as higher estrogen and progesterone and lower testosterone levels than males. Moreover, females had higher glutamate turnover ratios in the hippocampus and prefrontal cortex, and lower prefrontal GABA levels, compared with males.

In the present study, locomotor and exploratory activity were measured with the open field, light/dark box, and hole-board tests (Kafetzopoulos et al. 2018; Patel et al. 2005), and an interesting effect of BNN27 was revealed. In the open field test, acute administration of the BNN27 high dose (90 mg/kg) markedly suppressed spontaneous locomotion in both sexes, both in terms of horizontal distance and vertical rearing behavior. Therefore, the high BNN27 dose (90 mg/kg) was not used in the subsequent behavioral tests, due to its pronounced sedative effects in the open field test. Effects of BNN27 on locomotion were corroborated by the light/dark box test, where the intermediate 30 mg/kg dose of BNN27 significantly reduced total transitions but did not affect the time in the light compartment. On the other hand, it appears that acute administration of a low dose of BNN27 (10 mg/kg) does not affect locomotor activity. This is in accordance with a

previous study, where the total exploratory activity in the novel object recognition test was not affected by much lower BNN27 doses (3 mg/kg and 6 mg/kg) (Pitsikas and Gravanis 2017). Interestingly, BNN27 effects on locomotion have also been assessed in a schizophrenia model, where the same low doses tended to attenuate ketamine-induced hyperlocomotion (Zoupa et al. 2019). Notably, the decreased spontaneous locomotor activity is not reflected in altered levels of GABA and glutamate, suggesting a different mechanism for this effect.

The more complex hole-board test assesses aspects of anxiety and, possibly, memory, in addition to locomotion and exploration (Kliethermes and Crabbe 2006; Labots et al. 2015). Females were overall more active and exploratory than males, as measured by total number of hole explorations in the hole-board, and in line with our observations in the open field and the L/D box tests. They were also not affected by BNN27 administration. On the contrary, both 10 and 30 mg/kg BNN27 doses reduced the total number of holes explored by males and increased the latency time to explore the first hole. This effect could be due to decreased locomotion and exploration, or it could also indicate a possible anxiogenic effect (Calabrese 2008; Kumar et al. 2013; Takeda et al. 1998) of BNN27 in this test. However, neophilia, the driver of head dipping behavior, has been questioned as a pure index of anxiety, and head dipping behavior itself has been suggested to be an escaping behavior and/or reflecting altered locomotion (Brown and Nemes 2008; Kliethermes and Crabbe 2006). Moreover, this finding of possible anxiogenic properties of BNN27 in the hole-board test was not replicated in the other two tests (open field and light/dark box test); therefore, it should be interpreted with caution and warrants further exploration. On the other hand, the lower hole-board working memory index in both BNN27 male treatment groups signifies less repetitive exploration of the same holes, possibly due to an improvement in short-term working memory, whereas the same index was not changed in treated females. However, the current hole-board design did not include baits and rewards, which are commonly used in modified settings in order to better assess memory (Labots et al. 2015; Sampedro-Piquero et al. 2019). Confounding by other drug effects (increased arousal, attention, or sensorimotor activity) is unlikely based on the unchanged total exploratory activity in the low BNN27 doses (Pitsikas and Gravanis 2017; Zoupa et al. 2019). The effect of BNN27 on memory was previously studied with more reliable tests in the ketamine-induced psychosis model (Zoupa et al. 2019), where BNN27 protected from non-spatial and spatial memory deficits (cognitive symptoms). Our study did not aim at directly assessing cognitive effects of BNN27, and the repetition index is only a crude estimate of working memory (Karl et al. 2006; Lin et al. 2010; van der Staay et al. 2012) in this single session hole-board without prior training in finding holes baited with food. In any case, however, the present finding is in line with recent findings on cognitive improvement following low doses of BNN27 in male rodents (Pitsikas and Gravanis 2017; Zoupa et al. 2019).

The forced swim test was used to assess the antidepressant potential of BNN27, which was negative in all behavioral parameters measured. Notably, we have recently reported that head shake frequency is mainly dependent on testosterone (Kokras et al. 2017), and thus the lack of BNN27 treatment effect validates the previous evidence that it also lacks estrogenic or androgenic effects (Calogeropoulou et al. 2009). However, this is only true for the low dose of BNN27 (10 mg/kg), as the higher dose of 30 mg/kg exerted a decrease in male testosterone levels. This decrease in testosterone levels could be linked to the decrease in transitions in the L/D paradigm, suggesting a decrease in general locomotion, which is known to be affected by testosterone levels and castration in male rats (Kokras et al. 2018). Testosterone levels have not been measured before in the context of BNN27 administration, and this should be further studied. Possible explanations could involve the increased progesterone levels or estrogen antagonism in high doses. Notably, BNN27 has been designed with a modification at C17 that expressly prevents metabolism into androgens or estrogens. BNN27 did not affect estrogen levels, validating previous evidence that this synthetic microneurotrophin is not metabolized into estrogen (Calogeropoulou et al. 2009). Another interesting observation is that intermediate doses of BNN27 increased progesterone levels in both sexes. It has been shown previously that increased progesterone results in reduced glutamate levels in human blood (Tsesis et al. 2013; Zlotnik et al. 2011). DHEA, the natural precursor of BNN27, has also been shown to increase progesterone levels when administered during in vitro fertilization treatment in women (Weissman et al. 2011). The underlying mechanism is believed to involve increased adrenal progesterone synthesis (Strauss et al. 2014). The possibility that BNN27 shares this property with DHEA requires further investigation. However, it should be noted that hormone measurements were performed with radioimmunoassays in serum extracts without pre-purification steps and it is not clear whether non-specific binding took place.

In the hippocampus, BNN27 did not affect glutamate levels, whereas only the low BNN27 dose increased GABA and glutamine levels in both sexes. The increased GABA levels do not necessarily correspond to increased GABAergic neurotransmission or hippocampal hypofunction. Interestingly, high doses of DHEA, the parent molecule of BNN27, can act as a negative modulator of GABAergic neurotransmission (Gartside et al. 2010). This finding could also represent generalized hippocampal hyperactivity, particularly in combination with increased glutamate activity (Patel et al. 2005). Indeed, the hippocampal glutamate turnover rate, as measured by the glutamine/glutamate ratio, significantly

increased upon administration of BNN27 low dose but did not reach significance in the high dose group. Moreover, in the prefrontal cortex, GABA and glutamine were unaffected, but male rats treated with the low BNN27 dose had higher glutamate levels in the prefrontal cortex. It is interesting to note that all these neurochemical effects of BNN27 were observed in the lower, but not higher BNN27 dose, and this result should be interpreted cautiously. They could possibly be explained by a bell-shaped dose-response curve. Nevertheless, increasing GABA levels in the hippocampus could be an indicator of an anxiolytic effect, although the present behavioral findings do not support this hypothesis (Holm et al. 2011). With regard to correlation of behavioral tests with neurochemistry, previous experiments by our group have shown that the head shake behavior correlates with dopamine, serotonin, and amino acids in the PFC, but not the hippocampus (Kokras et al. 2018; Mikail et al. 2012). Moreover, both the prefrontal cortex and the hippocampus are key players in the stress and anxiety behaviors, as anxiety in the light/dark box test is mediated by the ventral hippocampus (Riaz et al. 2017), whereas hole-board test anxiety is mediated by the PFC (Goes et al. 2018). It should also be noted that locomotion, anxiety, and brain neurotransmitter levels were examined at only one timepoint, 30 min after BNN27 administration. Possible time-course effects were not studied herein and could be examined in future experiments.

Overall, BNN27 does not seem to exert anxiolytic or antidepressant effects, which is consistent with a DHEA clinical trial in patients with schizophrenia, where DHEA improved negative symptoms but not depressive symptoms or anxiety (see Cai et al. 2018 for a comprehensive table). It further confirms that BNN27 affects the glutaminergic and GABAergic systems, as does its parent molecule, DHEA (Bergeron et al. 1996; Majewska 1992). Low BNN27 doses increased glutamate-to-glutamine cycle activity (turnover), possibly affecting the activity of glial glutamine synthetase, an important step in the glutamate-glutamine cycle. However, GABA is also an important player in this cycle, representing 23% of the total glutamate+GABA-to-glutamine cycling (Patel et al. 2005). Therefore, changes in the turnover could represent altered GABA turnover, as well. Present findings could be related to beneficial effects of BNN27 in rodent models of neurologic disorders. BNN27 has shown beneficial behavioral effects in an ALS model (increased time spent on rotarod and paw grip endurance), albeit in female mice only (Glajch et al. 2016). This gender-based difference can be attributed not only to sex differences in the disease course but also to a possible interaction of high, continuous BNN27 concentration with estrogen receptors (Glajch et al. 2016), as BNN27 is a weak ERβ antagonist (Calogeropoulou et al. 2009). It also exerts a trophic action on oligodendrocytes, reduces microglia activation and inflammatory response, and increases neuronal survival in the experimental autoimmune encephalomyelitis model of multiple sclerosis (Bonetto et al. 2017). Additionally, BNN27 showed protective properties in a retinal detachment model (Tsoka et al. 2018) and appears promising in Alzheimer's disease models (Pitsikas and Gravanis 2017). More recently, BNN27 was also found to have protective effects in a rat schizophrenia model which involves NMDA blockade by ketamine, where BNN27 attenuated hyperlocomotion, reduced ataxia (positive symptoms), reduced social isolation in high doses (negative symptom), and protected from non-spatial and spatial memory deficits (cognitive symptoms) (Zoupa et al. 2019). It was inferred that BNN27 exerts these psychotropic effects through interaction with the NMDA and sigma-1 receptors, as well as its antioxidant and anti-inflammatory effects (Zoupa et al. 2019). The present study also confirms that BNN27 interacts with the glutamate-glutamine cycle.

Sex differences were also evaluated in the present study, as they should be always taken into consideration, not only with regard to the epidemiology of psychiatric disorders but also in the behavioral outcomes of preclinical models (Kokras and Dalla 2014) and in molecular pathophysiology involving neurosteroids and other neurotrophic factors. In the present study, an interesting sex difference in treatment effect was observed in the hole-board test, as well as in glutamate levels in the PFC: BNN27 reduced total number of holes explored, as well as the hole-board index, only in males, but not in females, which can be translated to decreased locomotion and, possibly, improved short-term memory in males, but not females treated with BNN27. In parallel, the intermediate BNN27 dose enhanced glutamate levels in the male PFC only. Other sex differences identified in this study concern findings regardless of treatment effect and they are in accordance with the relevant literature. In the open field test, females had significantly higher activity (horizontal and vertical counts), which represents a known sex difference (Kokras and Dalla 2014; Kokras et al. 2018). In the light/dark box test, females had higher total transitions and time spent in the light compartment (Kokras and Dalla 2014). In the hole-board test, females were again more active and exploratory, as measured by the total number of hole explorations, and this sex difference is in accordance with previous observations (Brown and Nemes 2008; Kokras and Dalla 2014). Finally, at the forced swim test, females had lower head shaking counts, which also agrees with previous evidence and could be linked to lower testosterone levels (Kokras et al. 2017; Kokras and Dalla 2014). Moreover, females had lower glutamate levels in the hippocampus and prefrontal cortex, and lower GABA levels in the prefrontal cortex

compared to males. Glutamate turnover was higher in females, both in the hippocampus and PFC. The present findings, in combination with sex differences identified in previous studies (Kokras et al. 2009; Kokras et al. 2018), highlight the need for the elucidation of sex differences in amino acid neurotransmission.

Conclusion

The promising neuroprotective effects of BNN27 will, hopefully, soon lead to its testing in human patients and, eventually, incorporation in neurologic disease therapeutics. Therefore, a detailed exploration of its pharmacodynamics, particularly with regard to mood and cognition, is imperative and has already begun in preclinical models. Moreover, the pro-cognitive effects revealed by similar studies may lead to its incorporation in the treatment of neuropsychiatric disorders. Finally, these experiments can shed light on the pathophysiology of these disorders and particularly on the role of endogenous neuroactive steroids and neurotrophins in their pathogenesis. The present study demonstrates that BNN27, like DHEA, seems to affect the glutamatergic and GABAergic systems. Current behavioral evidence does not support anxiolytic or antidepressant potential for BNN27, but in high doses, it can apparently lead to reduced locomotion and, possibly, sedation. More detailed preclinical dose-dependent studies in both sexes are required.

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Author contributions NK was involved in all stages of the experimental procedures, data collection, analysis, and interpretation. FD and KA provided insights regarding the behavioral experiments. C. Dioli performed behavioral experiments. RP performed neurochemical measurements. AG, IC, and TC provided the BNN27, as well as gave valuable advice and critically reviewed the manuscript. MGS assisted in the data and manuscript preparation. C. Dalla supervised the project and manuscript preparation.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

All rats were handled in accordance with the guidelines for the care and handling of laboratory animals in EU Directive 2010/63 and the experiments were approved by the local committee (Prefecture of Athens, Greece, protocol number 3288/15 May 2012).

Conflict of interest NK and CD have received honoraria and financial support from Janssen-Cilag, Elpen S.A. and Medochemie S.A. MGS has received financial support from Mallinckrodt. None of those is relevant to this study. AG is the co-founder of the spin-off Bionature EA LTD, proprietary of compound BNN27 (patented with the WO 2008/1555 34 A2 number at the World Intellectual Property Organization).

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