**Methods**

**according to the ARRIVE Guidelines (**<https://doi.org/10.1371/journal.pbio.1000412>**)**

|  |  |
| --- | --- |
| Ethical statement | The different experimental groups and procedures were performed in conformity with the Brazilian Council for the Control of Animals under Experiment (CONCEA), which are in compliance with international laws and politics. The local Ethical Committee approved the experimental protocol (number 08.1.233.53.5).  |
| Study design | The number of experimental and control groups: **Behavioral Experiments**: Drug effects were evaluated in stressed (exposed to PT) and a non-stressed (exposed to habituation on shuttle box) groups in three different treatment schedules: **1.** **Repeated treatment**: animals were submitted to PT or habituation (day 1) and received one daily injection of 7-NI (30 mg/Kg), imipramine (15 mg/Kg) or vehicle (1 mL/Kg) for seven days. One hour after the last injection, all animals were exposed to LH testing (day 7); **2. Acute treatment on day 1**: animals were submitted to PT or habituation (day 1) and, immediately after, they received one injection of 7-NI (30 mg/Kg) or vehicle (1 mL/Kg) and returned to their home cages. On days 2 to 7, animals received one daily injection of vehicle (1 mL/Kg) and they were submitted to LH testing one hour after the last injection (day 7). **2. Acute treatment on day 7:** animals were submitted to PT or habituation (day 1) and received daily injections of vehicle (1 mL/Kg) on the following days (2,3,4,5 and 6). On the 7th days, animals received an injection of 7-NI (30 mg/Kg) or vehicle (1 mL/Kg) and were submitted to LH testing one hour later. Each experimental session included at least 2 animals of each treatment group and the experiment was repeated 4-5 times, always including animals of the different treatment conditions, until an appropriate number of animals in each group has been completed (13 per group according to G-power calculation). This approach aimed at including animals of all groups in the same experimental day and also to avoid possible environmental influences on individual behavioral analysis conducted in a single day. **BDNF analysis**: independent groups received daily injections of 7-NI (30 mg/Kg) or imipramine (15 mg/Kg) or vehicle (1 mL/Kg) during 7 days and sacrificed for hippocampus dissection one hour after the last injection. An additional experimental group received daily injections of vehicle for 6 days and single injection of 7-NI (30 mg/Kg) or imipramine (15 mg/Kg) or vehicle (1 mL/Kg) on the 7th day and were sacrificed one hour later for hippocampus dissection. As described for behavioral analysis, all treatment groups were carried out in the same experimental session, including 4 animals/treatment group and the experiment was repeated once to achieve 8 animals/group.The table represented bellow describes the experimental design and experimental groups carried out in the present work.Control groups: In order to control treatment effects all treated groups were carried out together and tested in the same experimental session, which thus included 7-NI, imipramine (positive control) and vehicle (drug control group). Additionally, drug effects in non-stressed groups were evaluated to investigate possible drug-induced effects per se which would be unrelated to stress exposure. |
| Experimental procedures | Learned Helplessness: The behavioral procedure consisted of two experimental sessions conducted in day 1 (pre-test or habituation) and in day 7 (test). The animals in the pre-test group (PT) were submitted to 40 inescapable footshocks (1mA, 10 s duration) given according to a variable time schedule with a mean interval of 60 s (range from 30-90s). The rats in the habituation group were placed into the shuttle box apparatus for 30 minutes but no shock was given during this time. Six days later, animals of both groups were placed individually into the shuttle box and submitted to the test session (T). The test consisted of 30 escapable footshocks (0.8 mA, 10 sec duration, 30-90 sec interval) preceded by a tone (60dB, 670 Hz) that started 5 seconds before each shock and lasted until the end of the footshock. Animals were tested at random order along the day to avoid possible circadian influences on behavior. All experiments were conducted between 8:00 am and 4:00 pm.Protein extraction: The animals were deeply anaesthetized (chloral hydrate 5%, 10 ml/kg) and sacrificed one hour after the last injection. Their hippocampi were dissected and homogenized in lysis buffer (NaCl 137 mM; Tris-HCl 20 mM pH 7.6; glycerol 10%) containing protease inhibitor cocktail (Cat# P2714, Sigma, USA). After centrifugation (20000 g, 15 min), the supernatant was collected and stored at -80ºC until use. Bradford method: An aliquot of each sample was reserved and used to determine the total proteins levels using the Bradford method (Bradford 1976). Briefly, 50 μL of diluted sample and 170 μL of Bradford Reagent (Sigma Aldrich, #B6916) were added to each well of a microplate. The optical density was quantified at 595nm. Total protein values in μg/μL of sample were calculated based on the standard curve of bovine serum albumin (BSA; Sigma Aldrich, #P0914), which ranges from 0.031 to 0.500 μg/μL.ELISA method: Hippocampal BDNF was measured by ELISA (Cat# G7610, BDNF Emax ® ImmunoAssay System kit, Promega, USA) according to the manufacture’s instructions. Briefly, 96-well plate was precoated with a primary monoclonal antibody against BDNF overnight at 4oC. Following 1h blockade with BSA solution, supplied by the kit, we added the samples (2h incubation at room temperature) and later the secondary polyclonal antibody (2h incubation at room temperature). After incubating for 2h at room temperature with the tertiary HRP-conjugated, colorimetric detection of peroxidase activity was achieved by adding tetramethylbenzidine (TMB One) solution. The enzymatic reaction was stopped with hydrochloric acid (HCl) 1M and the color intensity of each well was measured at 450nm using a plate reader (VictorX3, Perkin Elmer, USA). A standard curve was generated using values from the dilution series of a recombinant human BDNF standard (also supplied by kit) and was used to determine the BDNF concentration. Data was normalized by total protein levels in each sample.Drug administration: Regarding drug choices, drug doses and route of administration, our choices were based on previously published papers and experience from our research group in order to test our hypothesis. In Figure 1, two doses were tested in animals submitted to learned helplessness model and the dose of 30mg/Kg had effect, so this dose was used in all experiments. **Figure 1: Effects of the treatment with imipramine (imi, 15mg/Kg) and 7-NI (30 or 60 mg/kg) in animals submitted to learned helplessness model.** Rats submitted to the pre-test (inescapable footshocks) or habituation (no shocks) were treated for 7 days with imipramine (15mg/Kg) or 7-NI (30 or 60mg/kg) and then submitted to learned helplessness test (escapable footshocks). The graph represents the percentagem of escape failures (mean±sem), n = 6-20 animals/group. (\*p<0,005).  |
| Experimental animals | Male Wistar rats weighing 200-220g at the beginning of each experiment were individually housed in Plexiglas cages and kept in a temperature-controlled room (24 ± 1oC), under standard laboratory conditions with free access to food and water and a 12h light/12h dark cycle (lights on at 06:30h a.m). Animal source: All animal used came from a breeding facility from the University of São Paulo – Campus Ribeirão Preto.Facilities and nutrition: The animals were kept in a non-SPF facility inside the laboratory. The housing cages had no environmental enrichment. The animals had access to tap water and the food was delivered in processed chew pellets (Nuvilab, CR-1, Brazil) containing (as informed by the provider): SODIUM CHLORIDE (SALT COMMON), VITAMIN A, VITAMIN A, VITAMIN D3, VITAMIN E, VITAMIN K3, VITAMIN B1, VITAMIN B2, VITAMIN B6, VITAMIN B12 , NIACINE, CALCIUM PANTOTENATE, FOLIC ACID, BIOTINE, HYDROCHLORIDE CHLORIDE, MANGANESE MONOXIDE, ZINC OXIDE, COPPER SULFATE, CALCIUM IODATE, SODIUM SELENITE, COBALT SULFATE, LYSINE, METHIONINE, BHT. GUARANTEE LEVELS BY PRODUCT KILOGRAM: HUMIDITY (MAX) 125 G / KG; MINERAL MATERIAL (MAX) 90 G / KG; CALCIUM (MIN-MAX) 10-14 G / KG; GROSS PROTEIN (MIN) 220 G / KG; GROSS FIBER (MAX) 70 G / KG; PHOSPHORUS (MIN) 8,000MG / KG; EXTRACT ETEREO (MIN) 40 G / KG; VITAMINS: VITAMIN A (MIN) 13,000 IU / KG; VITAMIN D3 (MIN) 2,000 IU / KG; VITAMIN E (MIN) 34 IU / KG; VITAMIN K3 (MIN) 3 MG / KG; VITAMIN B1 (MIN) 5 MG / KG; VITAMIN B2 (MIN) 6 MG / KG; VITAMIN B6 (MIN) 7 MG / KG; VITAMIN B12 (MIN) 22 MCG / KG; NIACINE (MIN) 60 MG / KG; CALCIUM PANTOTENATE (MIN) 20 MG / KG; FOLLIC ACID (MIN) 1 MG / KG; BIOTINE (MIN) 0.05 MG / KG; HILL (MIN) 1,900 MG / KG. MINERALS: SODIUM (MIN) 2,700 MG / KG; IRON (MIN) 50 MG / KG; MANGANES (MIN) 60 MG / KG; ZINC (MIN) 60 MG / KG; COPPER (MIN) 10 MG / KG; IODO (MIN) 2 MG / KG; SELENIUM (MIN) 0.05 MG / KG; COBALT (MIN) 1,5 MG / KG; FLUOR (MAX) 80 MG / KG. AMINO ACIDS: LYSINE (MIN) 12 G / KG; METHIONINE (MIN) 4,000 MG / KG. ADDITIVES: BHT 100 MG / KG. 3.485 Kcal/g.Welfare-assessments: The welfare of the animals was assessed daily. The cages and bedding were changed every two days as well as food and water replacement. |
| Housing and husbandry | Behavioral Experiments: The animals were received from breeding facility (USP University Campus) and were maintained in the animal house of the Pharmacology Department for 7 days before starting experiments. Animals were kept in groups of four animals/cage (41cm x 34cm x 16cm) for at least one week until the beginning of the experiment. For behavioral experiments, animals were brought to the lab on day 1 and taken individually to experimental rooms, where they were exposed to PT or habituation. Immediately after completion of habituation or PT, animals received the administration of the drug or its vehicle. After that, they were housed individually in smaller cages (30cm x 19cm x 13cm) and taken back to the animal house. Every day, at 7:00 am, the animals were brought to the lab experimental rooms, where they were weighted and injected with drug or vehicle, according to the groups they had been randomly assigned to and then taken back to the animal room. The animals assigned to BDNF analysis were maintained in the animal house at least one week until the beginning of the treatment procedure, under the same conditions described above for animals submitted to behavioral experiments. On first day of treatment (day 1), animals were brought to the lab, where they were weighted and randomly assigned to the different treatment groups. After injection of vehicle, imipramine or 7-NI, they were housed individually in smaller cages (30cm x 19cm x 13cm) and taken back to the animal house. Every day at 7:00 am the animals were brought to the lab, where they were weighted and injected with drug or vehicle, and then taken back to the animal keeping room. On last day (7th), the animals received the last injection and returned to their boxes where their remained undisturbed for one hour. After that time, they were anaesthetized and sacrificed for hippocampus dissection. The last treatment injection and sacrifice were performed in random order to avoid circadian influences on BDNF analysis in individual treatment groups.  |
| Sample size | Sample size: The probable number of animals per group was calculated using the program G\*power 3.1.9.2 in which it was selected the following options for each field: “F test” 🡺 “ANOVA: fixed effects, special, main effects and interactions” and, on the type of power analyses, we choose the option “A priori: compute required sample size – given α, power, and the effect size. So, based on previous results, we determine the effect size f is 0.15, the α error probability is 0.05 and the power (1 – β error probability) is 0.20, the numerator df is 2 and the number of groups is 6. Based on that, the sample size required for LH experiments is about 13 animals per group. For the learned helplessness experiments we had two experimental conditions (stressed and non-stressed) that received repeated treatment with different drugs (control, imipramine and 7-NI). Besides that, we had the groups stressed and non-stressed that were acutely treated with 7-NI or vehicle in two different moments (on day 1, after PT and on day 7, before T). For BDNF analysis, 8 animals were used in each group. The total number of animals used was 175 male Wistar rats, and this number is close to the number predicted by G\*power program (204 animals). It is important to highlight that in some experimental groups the number of animals was smaller than expected due to the following exclusion criteria: animals that presented no crossing during test session, inflammation in the site of injection or other health issues. |
| Allocating animals to experimental groups | Group randomization and analyses: Animals were randomly assigned to the different experimental conditions (stressed or non-stressed) and treatments groups (7-NI, Imipramine or vehicle) by individual sorting. The different treatment groups were also tested (or sacrificed, in the case of BDNF analysis) at random order on the experimental session to avoid possible circadian influences on behavioral and molecular analysis. Behavioral changes were recorded as failures to escape/avoid shocks and number of intertrial crossings, which were automatically registered by the shuttle box Automatic Reflex Conditioner no 7502 – UGO BASILE Biological Research Apparatus program. Animals that presented abnormal locomotion during the test session (i.e.: presented no crossing behavior in the shuttle box), regardless of their treatment group, were excluded from statistical analysis.  |
| Experimental outcomes | The first experimental outcome assessed was the behavioral changes measured by the number of intertrial crossing and escape failures in animals submitted to learned helplessness model. Independent groups were submitted to pharmacological treatment and sacrificed to analyze molecular markers that, in this case, were BDNF levels in the hippocampus. |
| Statistical methods | The total number of inter-trial crossings (ITC) and escape failures were calculated for each animal and analyzed by Kruskal-Wallis test. BDNF levels were analyzed by one-way ANOVA followed by Dunnett’s Multiple Comparison test. Probability less than 0.05 was accepted as significant. |