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Anticompulsive-like effect of nitric oxide synthase inhibitors in marble-burying test

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Abstract

Nitric oxide synthase (NOS) inhibitors decrease marble burying behavior (MBB), and the effect of several compounds that also attenuate MBB (such as classical antidepressants) engages the nitrergic system. In the present study, we tested the effect of the NOS inhibitor aminoguanidine (AMG) in attenuating MBB. For comparative reasons, we also tested the effect of selective inhibitors of neuronal (NOS1) and inducible (NOS2) isoforms NPA and 1400W, respectively. Our results indicate that AMG and NPA, but not 1400W, reduced the number of buried marbles in the marble burying test (MBT), which is considered an anticompulsive-like effect. No effect of AMG in the anxiety- or locomotor-related parameters of the elevated plus maze was observed. Taken together, our data is consistent with the current literature that suggests that nitric oxide inhibitors, putatively acting through the neuronal isoform of the synthesis enzyme (NOS1), exhibit anticompulsive-like properties.

Keywords: marble burying test, nitric oxide synthase inhibitors, elevated plus maze, anxiety, repetitive behavior, NOS

Repetitive behaviors are prominent elements of the normal repertoire that animals acquire and develop at an early age. These stereotyped movements are regulated by a series of reverberatory loops in the cortico-striato-thalamo-cortical circuitry (1). Disruptions or imbalances (such as stressful situations or trauma) in the components of this circuitry lead to pathological conditions such as Parkinson's disease, autism, and obsessive compulsive disorder (2). Transgenic animals lacking docking proteins in cortico striatal glutamatergic synapses exhibit exacerbated repetitive movements, such as grooming and barbering (3,4).

Excessive glutamatergic transmission is associated with several stress-related disorders, such as anxiety (5), and triggers the production of the retrograde messenger nitric oxide (NO) by its synthase, NOS(6–8). For instance, increased production of NO follows exposure to stress (9).

Pharmacological inhibition of NOS1/NOS2 exerts antidepressant- (10,11), anxiolytic- (12), and anticompulsive-like effects (13). Here, we attempted to replicate the effect of NOS inhibitors in the marble burying test (MBT), a simple model of repetitive behavior in mice, sensitive to NOS inhibitors (13), using aminoguanidine, a non-selective NOS2/NOS1 inhibitor, in comparison with the more selective NOS1 inhibitor n^{ω}-propyl-L-arginine hydrochloride (NPA) and NOS2 inhibitor 1400W dihydrochloride. In order to control for a putative general anxiolytic-like effect, we also tested the effect of AMG in the elevated plus maze- EPM (14).

Adult male Swiss (12-18 weeks, 25-30g, total=81 animals) from the University of São Paulo, Ribeirão Preto Campus animal facility, were transferred to the School of Medicine vivarium 7

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days prior to the beginning of the experiments. The animals were group housed (10-12 animals per 30x20x12cm cage) under controlled temperature (24±1°C) with free access to food and water and a 12-h light cycle. All procedures were conducted in accordance with the Brazilian Society of Neuroscience and Behavior guidelines for the care and use of laboratory animals and were in compliance with international laws and policies. The local ethics committee approved the experimental protocol and all efforts were made to minimize animal suffering (protocol: 146/2009). The animals were left undisturbed in the experimental room for 1h prior to the experimental session. All behavioral analyses were conducted with independent cohorts of experimentally naive animals tested 30 min after administration of the following drugs: aminoguanidine hydrochloride (AMG, Sigma-Aldrich, #396494), nω-propyl-L-arginine hydrochloride (NPA, Tocris, #1200), and 1400W dihydrochloride (Tocris, #1415). This group of compounds are assumed to penetrate to the brain (15,16), and the doses used were based on previous studies in the literature (10). All drugs were dissolved in sterile saline (vehicle) and injected intraperitoneally (ip) at 10 ml/kg volume. All data were analyzed using GraphPad Prism (v.5) and are available in FigShare under CC-BY license (DOI:10.6084/m9.figshare.12206597).

The MBT was performed using a squared arena (30x20x12cm) containing a 5cm high sawdust layer. Twenty-five green clear glass marbles (1.5cm in diameter) were evenly spaced on top of the sawdust. The animals were randomly assigned to one of the experimental groups by one experimenter, received the injection, and 30min later, they were individually placed in the center of the arena. After 25 min of free exploration, the number of buried marbles was counted by another experimenter blind to the treatments. Marbles were considered buried if at least two-thirds of their surface was covered by sawdust (13,17–19). Given the nature of the independent variable, *i.e.* discrete, the data from MBT were analyzed using the Kruskal-Wallis rank test. Aminoguanidine (300 mg/kg) decreased the the number of buried marbles compared to the control group, an effect interpreted as anticompulsive-like; a similar outcome was observed by the selective NOS1 inhibitor NPA at 5.2µg, but not at 1.3µg or by the NOS2 inhibitor 1400W (0.75 or 1.3µg), figure 1A. Two animals were excluded from the data analysis (in the NPA 1.3µg group), due to leaking of the solution with blood observed following the drug administration.

The MBT was initially proposed as a model of neophobia (17) and is indeed sensitive to benzodiazepines (17,18,20). However, contrary to other models sensitive to anxiolytics, the observed outcome (*i.e.* the number of buried marbles) does not decrease following re-exposure (17,20,21). This feature of MBT led to the proposal that the burying behavior reflects a natural, repetitive behavior that could become compulsive. In fact, there is no correlation between the outcomes of MBT and other anxiety-related measures, *i.e.* light-dark transitions (21).

Pharmacological studies employing MBT have described positive effects of ketamine and anti-glutamatergic agents (22–24), antidepressants (25–27), paroxetine (26,28), cannabinoids (20,29), and antipsychotics (30,31) in this model. Inhibitors of NOS1 are also effective in the MBT (13). Consistent with these findings, our results point to a reduction of MBB by AMG and NPA, suggesting that NOS1 may be the main isoform involved in the circuitry responsible for regulating MBB. Although a preferential inhibitor of the NOS2 isoform, AMG is probably also able to inhibit NOS1, given that its affinity for NOS2 over NOS1 is only 4.7-fold higher (32). Moreover, in healthy naive animals NOS2 is minimally expressed, while NOS1 is constitutively expressed in the brain (10,33). Interestingly, the nitrergic neurotransmission also seems to be crucial for the effect of compounds with putative different mechanisms of action. The previous administration of the NO precursor L-arginine prevented the effect of the serotonin uptake inhibitors paroxetine and citalopram in MBT (13,34) and the effect of agmatine, an NMDA receptor antagonist (35). Another study suggested an association between NO production, measured by levels of active NOS1, and the anticompulsive effects of the P2R antagonist iso-PPADS (36). Although the mechanisms are not clear, clinical data indicates that OCD patients' responsiveness to SSRIs is associated with a decrease in serum levels of nitrate/nitrite (37).

For the 5 min EPM session, an experimentally naive cohort of animals was randomly assigned to the drug treatments. At 30 min after the injection, individually placed in the center of the wood made apparatus, composed of two opposite open-arms (30cm x 6cm) and two enclosed arms (30cm x 6cm x 5cm), elevated 50cm above the ground. The percentage of entries and time in the open arm (%OAE, %OAT respectively) and the number of entries in the enclosed arm (EAE) were analyzed by the ANY-MAZE software (Stoelting, USA). According to the factorial analysis of this model for mice, the %OAE and %OAT are the parameters mostly associated with anxiety, while the number of entries in the enclosed arm (EAE) is related to locomotion (38). Given the nature of the variables (continuous), and since no difference was observed in Bartlet's or Brown-Forsythe's tests, the data from %OAT and %OAE were submitted to one-way ANOVA, while the data from EAE (discrete) were submitted to the Kruskall-Wallis test. In agreement with the distinction between MBB and general anxiety trait, we did not observe any effect of AMG in the EPM, as seen in figure 1B-D. Previous studies indicated that AMG only exerted anxiolytic properties in animals submitted to stress or under an anxiogenic stimulus (12,39).

In conclusion, the data reported in the present study support previous evidence that NOS inhibitors attenuate MBB, and this feature does not seem to be a consequence of the general anxiolytic effects.

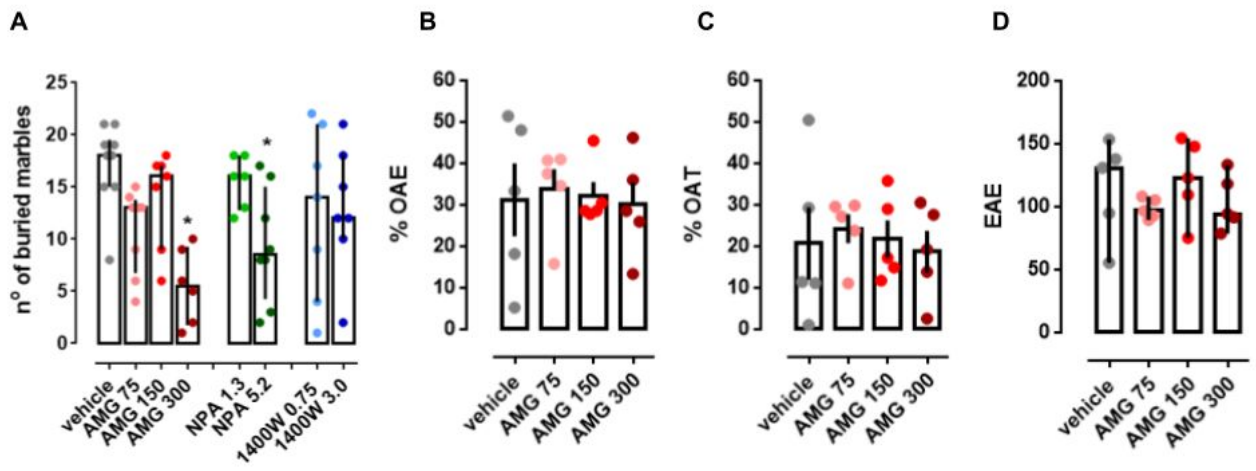

Figure 1. (A) Systemic administration of aminoguanidine and NPA exerts anticompulsive-like effect in the MBT test [treatment: Kruskal-Wallis H(7)=20.78; p=0.0041, n=10,8,7,6,6,8,7,7]. Aminoguanidine (AMG, 300 mg/kg), as well as the selective NOS1 inhibitor NPA (5.2µg/kg) reduced the number of buried marbles in MBT ($p<0.05$, Dunn's). No effect of the selective NOS2 inhibitor 1400W was observed. (B,C) AMG did not alter the EPM parameters related to anxiety, % open arm entries [$F(3,16)=0.0781$, $p=0.972$] and % open arm time [$F(3,16)=0.159$, $p=0.922$], or the (D) locomotor aspects of the model, enclosed arm entries [treatment: Kruskal-Wallis H(3)=2.669, p=0.446], n=5/group. Data from the number of buried marbles (A) or enclosed arm entries (D) are expressed as median ± interquartile range, data from % open arm entries (B) and % open arm time (D) are expressed as mean ± SEM. *$p<0.05$ from vehicle group.

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