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Short communication

L-NAME differential effects on diazepam and flunitrazepam responses of rats in the object recognition test

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ABSTRACT

Background: The present study was undertaken to better understand possible interaction(s) between a non-selective nitric oxide inhibitor: N^G-nitro-L-arginine methyl ester (L-NAME) and benzodiazepines (BZs) in recognition memory.
Methods: The study was carried out on adult male albino Wistar rats. A novel object recognition (NOR) task was used to evaluate memory process.
Results: Combined administration of L-NAME (50 mg/kg, *ip*) with a threshold dose of DZ (0.25 mg/kg) induced amnesic effects in rats, participating in the NOR test. On the other hand, following a combined administration of L-NAME (100 mg/kg, *ip*) with flunitrazepam (FNZ; 0.1 mg/kg), it was found out that L-NAME inhibited the amnesic effects of FNZ on rats in the NOR test.
Conclusions: The obtained results suggest that suppressed NO synthesis may lead to a facilitation of DZ-induced memory impairment but surprisingly may prevent amnesic effect after FNZ in rats, submitted to NOR task.
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Introduction

Benzodiazepines (BZs) are the most widely used anxiolytic drugs but they are also known to suppress the ability to learn and form new memories *i.e.*, BZs induce anterograde amnesia [1–3]. BZs-related memory impairment is mediated by the gamma-aminobutyric acid ($GABA_A$) receptor [1,3]. In addition, it is well documented that the amnesic action of BZs involves more subtle alterations in hippocampal synaptic transmission – plastic changes on cell membranes – *i.e.* long-term potentiation (LTP) [3]. Nonetheless, little is known about the precise brain mechanisms underlying BZs-induced behavioral deficits during episodic memory encoding. Moreover, an extensive research has been carried out, focusing on the influence of BZs on memory in mazes which are the experimental devices, often employed for evaluation of the spatial memory in rodents [1,4], but less is known about the BZ influence on the object recognition memory [2,3].

It has been shown that in humans BZs impair the episodic memory encoding which receives and stores information about temporally dated episodes and temporal–spatial relations among these events [2]. The investigation of episodic-like memory in animals refers to their ability to remember the "what" and "where" components of a unique episode, which can be assessed at the behavioral level using the novel object recognition (NOR) task [2,5]. The NOR is based on natural, exploratory abilities of rodents exposed to new objects [2]. In addition, object recognition is considered as hippocampal-independent task, while mEPM task (used in our prior studies) is a procedure sensitive to hippocampal function [5].

Nitric oxide (NO) has been proved to function as a novel retrograde intracellular messenger in the central nervous system (CNS) [6]. It is synthesized from L-arginine in a nitric oxide synthase (NOS)-catalyzed reaction. There are four members of the NOS family: neuronal (nNOS), endothelial (eNOS), inducible (iNOS) and mitochondrial (mtNOS) [6]. The role of NO in memory processes has broadly been examined and there is an overwhelming evidence for some involvement of the L-arginine:NO pathway in memory and learning processes [5]. Recent data have indicated some relationship between NO – and the GABA-mediated transmissions in the CNS [6,7]. A number of studies suggest that NO plays a modulating role in the neuronal release of GABA (for review, see [6]). Moreover, our previous behavioral studies demonstrated some interactions between the different effects of BZs and NO modulators. For instance, Talarek et al. [8,9] showed NOS inhibition as a prolonging factor of BZs-induced sleep time, also enhancing the anticonvulsant and antinociceptive effects of

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BZs. In addition, the results of our previous experiments also suggest that suppressed NO synthesis enhances diazepam (DZ)-induced, but prevents flunitrazepam (FNZ)-induced memory impairment, as confirmed in the modified elevated plus maze (mEPM) test on mice [10] and the NOR test in rats [11].

It should also be underlined that current literature data refers to conflicting results concerning the role of NOS inhibitors on memory process. The exact borderline between neuroprotective and pathological actions of NO is a matter of controversy among researchers in the field [12].

Regarding to the above, somewhat controversial results, the present study was aimed at better understanding of possible interactions between NO activity and responses, elicited by BZs in the NOR task in rats. For that purpose, L-arginine-analog inhibitor of NOS, N^G-nitro-L-arginine methyl ester (L-NAME) was chosen. The use of L-arginine-analog inhibitors of NOS has been very important in elucidation of the role of NO in various CNS physiological and pathophysiological parameters. The most commonly used inhibitor for the CNS is L-NAME, a non-selective NOS inhibitor which can inhibit both constitutive NOS: neuronal NOS (nNOS) and endothelial NOS (eNOS) [12]. In addition, it has been found that nNOS and eNOS are expressed in brain areas, including the cerebellum and the hippocampus, important structures for memory and synaptic plasticity [6].

L-NAME is a reversible NOS inhibitor but it appears to dissociate relatively slowly from NOS and has longer half-life (approx. 20 h) compared to 7-NI. 7-NI is considered to be a moderately weak and short-lasting inhibitor. Therefore, treatment with unselective inhibitor like L-NAME may ensure strong and long-lasting inhibition of NO formation. Indeed, many of the reported researches converge to indicate that the extent of the inhibition of NO production is even more critical than the specific source of production blocked in order to determine the final result of the pharmacological treatment (for review see [12]).

Moreover, there is now a little evidence claiming qualitative differences in the psychological effects and pharmacological properties of different BZs (for review see [13]). For instance, FNZ is suggested to differ from classic BZs. Hauser et al. [14] reported that FNZ can act as either an agonist or an inverse agonist, depending on $GABA_A$ receptor configuration. Additionally, it is known that its hypnotic and amnesic effects predominate over the sedative, anxiolytic and muscle-relaxant effects of other compounds from the same pharmacological group. These activities have also promoted its abuse as a date rape drug. It has been banned in the USA due to certain properties, such as severe aggression, and anterograde amnesia. However, it is still used in approximately 60 countries in psychiatry (anxiety, insomnia) or in anesthesia. It should be noted that FNZ is 10 times as potent as DZ – a drug with typical BZs effects [15]. In addition, most of our previous investigations on interactions NO and BZs showed no differences between BZs compounds *e.g.* DZ, chlordiazepoxide and clonazepam [8,9]. Considering these results, recent studies with a comparison of DZ and FNZ [10,11] seem to be very intriguing.

The aim of the present study was to compare mnemonic effects of two BZ compounds: FNZ (somewhat uncharacteristic) and DZ (a referent BZs drug) and to involve their interaction with L-NAME, a long lasting NOS inhibitor. This experiment is part of series of studies designed to evaluate to what extent NO modulators can affect BZs actions.

Material and methods

Animals

Two-month-old male albino Wistar rats (The Farm of Laboratory Animals, Z. Lipiec, Brwinow, Poland), weighing 200–250 g each, were used in the presented experiments. They were housed in groups of five per cage and maintained in a temperature-controlled room (21 °C) on a 12 h light-dark cycle. They received standard food (Agropol, Motycz, Poland) and tap water *ad libitum*. All behavioral experiments were carried out, according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and to the European Community Directive for the Care and Use of Laboratory Animals of 24th November 1986 (86/609/EEC), and approved by the Local Ethics Committee (37/2010).

Drugs

L-NAME and FNZ were purchased from Sigma Chemicals (St. Louis, USA). FNZ was dissolved in 0.5% Tween-80 (1–2 drops), gently warmed and diluted with saline solution (0.9% NaCl). DZ (Relanium, Polfa, Poland) was diluted in 0.9% saline. L-NAME was dissolved in saline solution. L-NAME was given intraperitoneally (*ip*), whereas DZ and FNZ subcutaneously (*sc*). All the drugs were injected in a volume of 0.2 ml per 100 g body weight. Control animals were administered a corresponding vehicle. The doses of administered substances were based on the protocols in previous experiments [10,11].

NOR test

The apparatus consisted of a square open box, made of plexiglass (63 cm long × 44.5 cm high × 44 cm wide) and illuminated with a lamp (light intensity – 10 lux), suspended 50 cm above the box. The objects to be discriminated, made either of wood or plastic, were in two different shapes: block and ball and they could not be displayed by rats.

The object recognition test was carried out as presented elsewhere [2]. This test included a period of habituation, an acquisition and a test trial. During a habituation (the day before the experimental day) each rat was placed in an empty box for 2 min. On the experimental day, the animals were submitted to two trials (a 1-hour interval). The first trial (acquisition trial, T1) lasted 5 min and the second one (test trail, T2) was 3 min long. During T1, two identical objects (wooden blocks) were put in two opposite corners, 10 cm from the sidewall. A rat was always placed in the middle of the box and was left to explore these objects. During T2, one of two similar objects, presented in T1, was replaced by a new object (N), therefore, the rats were re-exposed to two objects: the familiar one (F) and the new one (N). The exploration was defined as follows: directing the nose toward the object at a distance of no more than 2 cm and/or touching the object with nose. Turning around or sitting on the object was not considered as exploratory behavior. The time periods, spent by rats for exploration of each object during T1 and T2 tests, were manually recorded by a stopwatch. Recognition memory was evaluated, using the recognition index, calculated for each animal by the following formula: (N/N + F) × 100, corresponding to the difference between time exploration periods for novel and familiar objects, corrected for the total exploration time period of both objects. Higher values of the recognition index were considered to reflect stronger memory retention for familiar the objects [2]. In addition, in the results we report the total time (in s) exploring the two objects (N + F) during T1 and T2.

Treatment

L-NAME (50 and 100 mg/kg, *ip*) was administered alone, 35 min before T1. In order to evaluate the influence of L-NAME on DZ- or FNZ-treated rats, L-NAME was administered 5 min prior to DZ or FNZ injections.

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