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Research report

Effects of NOS inhibitors on the benzodiazepines-induced memory impairment of mice in the modified elevated plus-maze task

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HIGHLIGHTS

- Diazepam and flunitrazepam impaired memory in mice, in the elevated plus-maze task.
- NOS inhibitors prevented flunitrazepam-induced memory impairment.
- NOS inhibitors enhanced diazepam-induced memory impairment.

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ABSTRACT

The aim of the present study was to examine the effects of nitric oxide synthase (NOS) inhibitors on responses, elicited by benzodiazepines (BZs) in a modified elevated plus-maze task in mice. It was shown that acute doses of diazepam (DZ; 1 and 2 mg/kg) and flunitrazepam (FNZ; 0.05, 0.1 and 0.2 mg/kg) significantly increased the time of transfer latency (TL2) in a retention trial, thus confirming memory impairing effects of BZs. L-NAME (N^G-nitro-L-arginine methyl ester; 200 mg/kg), a non-selective inhibitor of NOS, and 7-NI (7-nitroindazole; 40 mg/kg), a selective inhibitor of NOS, further intensified DZ-induced memory impairment. On the other hand, L-NAME (50, 100 and 200 mg/kg) and 7-NI (10, 20 and 40 mg/kg) prevented FNZ-induced memory compromising process.

The results of this study indicated that suppressed NO synthesis enhanced DZ-induced but prevented FNZ-induced memory impairment. Taken together, these findings could suggest NO involvement in BZs-induced impairment of memory processes. The precise mechanism of these controversial effects, however, remains elusive.

1. Introduction

Benzodiazepines (BZs) constitute a group of psychoactive drugs, often used in the treatment of generalized anxiety, insomnia or convulsive disorders. It is also known that BZs disrupt memory, both in humans [1,2] and in animals [3–5]. BZs have repeatedly been found to induce temporary anterograde amnesia, affecting acquisition – the first stage of the memory process (new information encoding) [2,4,5]. An original (new) information has to first enter sensory channels (e.g., via visual, olfactory, auditory or tactile stimuli) to be then rapidly encoded into a form, transformable into short-term memory [6]. BZs act by enhancing the γ-aminobutyric $acid_A$ ($GABA_A$) receptor's function in the central nervous system (CNS). $GABA_A$ receptors are pentameric structures, derived from the assembly of various subunits and forming a channel through which chloride ions can pass. BZs have a separate binding site on these receptors and they are full agonists of $GABA_A$ receptors [7]. Many authors have indicated that BZs disrupt memory processes in result of their effects on $GABA_A$ receptors [8–10]. BZs binding sites are present on $GABA_A$ receptors at the CA1 region of the hippocampus (one of the brain structures, which is believed to play a critical role in memory processes) [8]. What is more, drugs, that act at the same binding site, however decreasing GABA's effects (inverse agonists), have been reported to improve cognitive functions [1,4]. Additionally, there are reports of BZs interference with long-term potentiation (LTP). For instance, BZs, when acting through hyperpolarisation cell membranes, would affect the generation of synaptic plasticity [5,8]. LTP is considered to be an important cellular mechanism, contributing to learning and memory processes [6].

Nitric oxide (NO), a free-radical gas, has been shown to exert various actions as a novel retrograde intracellular messenger in the CNS. NO is released in response to the activation of N-methyl-D-aspartate (NMDA) receptors in a nitric oxide synthase (NOS)-catalysed reaction [11]. There are four members of the NOS family: neuronal (nNOS), endothelial (eNOS), inducible (iNOS) and mitochondrial (mtNOS) but nNOS constitutes the predominant

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source of NO in neurons [12]. NO is proposed to play an important role in a series neurobiological functions, underlying learning and memory processes. For instance, some studies have been reported, indicating NO to be involved in LTP process [13]. In addition, NOS blockade by different inhibitors impairs animal performance in the object recognition paradigm [14], the 14-unit T-maze [15], the Y-maze task [16] and in the step-down passive avoidance task [17], as well as in the mEPM [18;19]. These deficits are antagonized by diverse NO donors: molsidomine [15] and L-arginine [17,19].

Literature data point to some relationship between NO- and GABA-mediated transmissions in the CNS [20–23]. A number of studies suggest that NO plays a modulating role in the neuronal release of GABA [20–22]. There have also been some lines of evidence for the co-localization of NO with GABA [23]. Recent studies have demonstrated that an activation of $GABA_A$ receptors by diazepam (DZ) increases the population of nNOS-positive cells in the frontal and the parietal areas of the developing cortex [24]. Our previous studies demonstrated NOS inhibition as a prolonging factor of BZs-induced sleep time [25], also enhancing the anticonvulsant [26] and antinociceptive [27] effects of BZs. Moreover, our previous findings suggested a certain role of NO in DZ-induced tolerance to its motor impairing and sedative effect in mice [28,29].

The aim of the present study was to design a possible interaction between NO activity and the responses, elicited by BZs in the modified elevated plus-maze (mEPM) task in mice. For that purpose, the effect of NOS inhibitors (N^G-nitro-L-arginine methyl ester; L-NAME and 7-nitroindazole; 7-NI) on DZ- and flunitrazepam (FNZ)-induced memory impairment was assessed in mice. FNZ was selected for this study because of its high ability to cause anterograde amnesia. FNZ is known as a date-rape drug, used by sexual predators to chemically incapacitate their victims [30]. DZ is a prototypical BZs, commonly used in clinical practice and can also produce anterograde amnesia [31].

It is known that L-NAME and 7-NI significantly reduce NOS activity in the rodent's CNS, after intraperitoneally (*ip*) administration [16,32]. However, L-NAME is a non-selective NOS inhibitor, therefore, it can cause marked hypertension, due to its effects on endothelial NOS [33]. It could be assumed that the vascular effect of L-NAME may have influenced memory and other behavioural performance [15,34]. On the other hand, NOS inhibitors injected systematically are supposed to induce a nearly maximal hypertensive effect at 10 mg/kg (we used higher doses of L-NAME in our experiments) [35]. What is more, it has been shown that L-NAME-induced hypertension did not alter either the inhibitory avoidance learning from the open arms in the elevated T-maze [36] or the transfer latency of rats in the mEPM [19]. Taken together, studies about vascular effect of L-NAME are not clear. Consequently, to avoid the non-specific effect of NOS inhibitor, 7-NI, a specific inhibitor for neuronal NOS, was also used. 7-NI, at doses up to 80 mg/kg, is reported to have no effect on the mean arterial pressure [37].

The mEPM task is a simple and not time-consuming model to evaluate spatial memory [38]. The procedure does not require manipulation of appetitive behavior such as good or water deprivation and the use of noxious stimuli such as electric shock or swimming stress [10,34]. The mEPM is based on the natural aversion, because during this mEPM exploration animals avoid the open and elevated spaces. Briefly, the time period, which the mice need to move from an open arm to an enclosed arm (transfer latency, TL), is used as the measure of learning and memory process performance. Prolongation of TL at the retention session confirms amnesic effects of the administered drug as the experimental animal does not remember the configuration of either the open arm or the enclosed arm. The drug administration prior to the first session may be used to determine its acquisition related actions [38,39]. What is more, mEPM has been successfully used, investigating the influence of different psychotropic compounds including BZs, on memory processes [39–44]. A systemic administration of BZs can induce memory (acquisition) impairment in different paradigms: the Morris water maze [45,46], the novel object recognition [5], the passive avoidance [3] and mEPM [39]. Therefore, in the reported experiment, animals were injected with BZ before the first test session.

2. Materials and methods

2.1. Animals

The examinations were carried out on male albino Swiss mice (Farm of Labolatory animals, Warsaw, Poland) weighing 20–25 g which were housed in groups of ten and maintained on a 12 h light-dark cycle at controlled temperature (21 °C). They received standard food (Bacutil, Motycz, Poland) and tap water ad libitum. All behavioural experiments were carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and to the European Community Directive for the Care and Use of Laboratory of 24 November 1986 (86/609/EEC), and approved by the Local Ethics Committee (37/2010).

2.2. Drugs

L-NAME and FNZ were purchased from Sigma Chemicals (St. Louis, USA). 7-NI (RBJ, Natick, USA) and FNZ were 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. All drug suspensions/solutions were prepared immediately prior to use. L-NAME, 7-NI and L-arginine were given *ip* whereas DZ and FNZ subcutaneously (*sc*). All drugs were injected in a volume of 0.1 ml per 10 g body weight. Control animals were given with the corresponding vehicle.

2.3. Modified elevated plus-maze test

2.3.1. Apparatus

The plus-maze was made of dark Plexiglas and consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) arranged such that two open arms were opposite to each other. The arms were connected by a central platform (10 × 10 cm). The apparatus was shaped like a "plus" sign and was elevated to a height of 50 cm above the floor [38–40]. The plus-maze was placed in a dark room illuminated only by a halogen lamp oriented towards the central platform and giving a uniform dim, red light in the apparatus (intensity of 60 lux).

2.3.2. Procedure

In the acquisition session (on day 1), each mouse was gently placed at the distal end of an open arms of the apparatus facing from the central platform. The time it took for the mouse to move from the open arm to one of the enclosed arms (transfer latency, TL 1) was recorded. If the mice failed to enter the enclosed arms within 90 s, they were placed at one of the enclosed arm and permitted to explore the plus-maze for additional 60 s. The criterion of an animal's entry into the enclosed arm was crossing with all four legs of an imaginary line separating the enclosed arm from the central space. The retention session followed 24 h after the acquisition session (on day 2). The mice were put into the one of open arms and the transfer latency (TL 2) was recorded again. If the mice did not enter the enclosed arm within 90 s the test was stopped. In such cases TL 2 was recorded as 90 s. TL 2 was utilized as an index of learning and memory processes. The prolongation of TL2 shows that drug has an amnesic effect while the shortage of TL 2 means that drug improves memory in mice relative to control groups [38–40].

The plus-maze was cleaned after each mouse. The experiments were conducted between 10:00 and 14:00 h.

2.4. Locomotor activity

The locomotor activity of individual mice was recorded using a photocell apparatus (round Plexiglas cage, 32 cm in diameter, Multiserv, Lublin, Poland). The cages were equipped with one row of infrared light-sensitive photocells (2 emitters and 2 sensors) located 1 cm above the floor. Locomotor activity was recorded by the number of photocell interruptions of each mouse for a total period of 10 min [47].

The animals were placed individually into cages, 30 min after the injection of DZ or FNZ and 35 min after the injection of L-NAME or 7-NI.

2.5. Treatment

2.5.1. DZ or FNZ effects on transfer latency of mice in the modified elevated plus-maze.

Different doses of DZ (0.5, 1, 2 mg/kg, *sc*) and FNZ (0.025, 0.05, 0.01 and 0.2 mg/kg, *sc*) were administered 30 min before the acquisition session (on day 1). Twenty-four hours later, a retention trial was performed in the same manner and transfer latency (TL2) was recorded.