



The effect of a selective neuronal nitric oxide synthase inhibitor 3-bromo 7-nitroindazole on spatial learning and memory in rats



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ABSTRACT

Since the discovery of nitric oxide (NO) as a neuronal messenger, its way to modulate learning and memory functions is subject of intense research. NO is an intercellular messenger in the central nervous system and is formed on demand through the conversion of L-arginine to L-citrulline via the enzyme nitric oxide synthase (NOS). Neuronal form of nitric oxide synthase may play an important role in a wide range of physiological and pathological conditions. Therefore the aim of this study was to investigate the effects of chronic 3-bromo 7-nitroindazole (3-Br 7-NI), specific neuronal nitric oxide synthase (nNOS) inhibitor, administration on spatial learning and memory performance in rats using the Morris water maze (MWM) paradigm. Male rats received either 3-Br 7-NI (20 mg/kg/day) or saline via intraperitoneal injection for 5 days. Daily administration of the specific neuronal nitric oxide synthase (nNOS) inhibitor, 3-Br 7-NI impaired the acquisition of the MWM task. 3-Br 7-NI also impaired the probe trial. The MWM training was associated with a significant increase in the brain-derived neurotrophic factor (BDNF) mRNA expression in the hippocampus. BDNF mRNA expression in the hippocampus did not change after 3-Br 7-NI treatment. L-arginine significantly reversed behavioural parameters, and the effect of 3-Br 7-NI was found to be NO-dependent. There were no differences in locomotor activity and blood pressure in 3-Br 7-NI treated rats. Our results may suggest that nNOS plays a key role in spatial memory formation in rats.

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1. Introduction

Some behavioural studies have shown nitric oxide (NO) to be involved in certain forms of memory formation. In rats, the systemic administration of nitric oxide synthase (NOS) inhibitors was found to produce learning deficits on the passive avoidance task (Komsuoglu-Çelikyurt et al., 2011; Utkan et al., 2012), to impair working memory in a three panel runway test (Utkan et al., 2012), and to induce delay-dependent performance deficits in the object recognition task (Pitsikas et al., 2003). Spatial learning has also been found to be impaired in rats after systemic administration of NOS inhibitors using the radial-arm maze (Böhme et al., 1993; Noda et al., 1997) and the Morris water maze (MWM) (Chapman

et al., 1992; Hölscher et al., 1995). In some cases of spatial learning, studies have shown conflicting results (Bannerman et al., 1994; Tobin et al., 1995). It is likely that these differences are most likely due to variations in dose, route of administration, and the nature of the compound studied. It is well documented that the predominant NOS isoform in neurons is nNOS, but there are several other brain regions that also contain endothelial nitric oxide synthase (eNOS) (Prast and Philippu, 2001). Both pharmacologic and genetic inhibition of nNOS has been shown to impair spatial performance in the MWM (Markvartova and Vozeh, 2008; Weitzdoerfer et al., 2004). Also, it is reported that stimulation of NO production from nNOS is necessary for the induction of long-term potentiation (LTP) in the hippocampus (Wu et al., 1997). 3-Br 7-NI is a more selective inhibitor of nNOS than the other two NOS isoforms, eNOS or inducible nitric oxide synthase (iNOS); therefore, 3-Br 7-NI is a more appropriate inhibitor to demonstrate the role of nNOS in the central nervous system (Bland-Ward and Moore, 1995). In our study, we have chosen to investigate the effect of a relatively specific nNOS inhibitor, 3-Br 7-NI, on spatial learning and memory. As peripheral administration of NOS inhibitors will affect the NO synthesis throughout the body and brain, it is difficult to determine which mechanisms are involved in the behavioural effects of NOS inhibition. Furthermore,

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NOS inhibitors have been found to produce several side effects, such as impaired locomotor activity and increased blood pressure that could interfere with normal behavioural functioning (Prendergast et al., 1997; Sandi et al., 1995). For these reasons, we chose to examine changes in blood pressure and locomotor activity as possible systemic effects of 3-Br 7-NI.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and is important in the modulation of synaptic transmission. BDNF enhances long-term potentiation (LTP) in the hippocampus (Figurov et al., 1996). Because LTP at the cellular level is involved in learning and memory, BDNF may be involved in memory processes. A relationship between BDNF mRNA expression and memory was shown in the water maze test (Kesslak et al., 1998), and spatial learning was impaired in BDNF mutant mice (Linnarson et al., 1997). In addition, learning-associated change in the BDNF mRNA level was observed only in the hippocampus (Mizuno et al., 2000). Therefore, we investigated the changes in hippocampal BDNF expression in 3-Br 7-NI treated rats.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Kocaeli University, Experimental Medical Research and Application Center, Kocaeli, Turkey) weighing 250–300 g were housed in groups of approximately 4 to 5 per cage. All experiments were conducted between 9:00 A.M. and 12:00 P.M. under standard laboratory conditions (22 ± 2 °C room temperature; 12-h light/dark cycle with lights on at 7:00 A.M.). Tap water and food pellets were provided ad libitum. All animals used in this study were naive to the experimental tests, and different rat groups were used in each experiment.

The experiments reported in this study were conducted in accordance with the Regulation of Animal Research Ethics Committee in Turkey (July 6, 2006, Number 26220). Ethical approval was granted by the Kocaeli University Animal Research Ethics Committee (Project number: HA EK 24, Kocaeli, Turkey).

Rats were divided into seven groups (the number of animals was 8 in each group): the first group received physiological saline (saline control), the second group received vehicle (5% DMSO), the third, fourth and fifth groups received 3-Br 7-NI (5, 10, or 20 mg/kg/day, i.p., respectively), the sixth group received the combination of 3-Br 7-NI (20 mg/kg/day, i.p.) and L-arginine (200 mg/kg, i.p.) and the seventh group received L-arginine (200 mg/kg, i.p.) alone. These doses were administered as single daily doses during 5 days.

All doses were prepared freshly in an injectible volume (0.1 ml/100 g body weight) and the doses were injected i.p. 30 min prior to training in the MWM daily, and L-arginine was injected 20 min prior to NOS inhibitor so that at the time of testing, the maximum effect of 3-Br 7-NI or L-arginine would be achieved, and acquisition of the task could be assessed (Komsuoglu-Çelikyurt et al., 2011; Utkan et al., 2012).

2.2. Behavioural assessment

The Morris task was assessed in a water tank (150 cm in diameter) as has been previously described (Pothion et al., 2004). The rats underwent three trials during five daily sessions. During the first four days, the platform, which was situated in the centre of the southwest quadrant, was submerged 1.5 cm below the surface of water, and small black pieces of plastic were placed on the water's surface. The plastic was invisible to the rats due to its placement, and it was used to monitor spatial learning. The platform position remained stable over 4 days, and acquisition of finding the platform was assessed. A trial was started by placing a rat into the pool, facing the wall of the tank. Each of three starting positions (north, east, and west) was used

once in a series of four randomly ordered trials. Each trial was terminated as soon as the rat had climbed onto the escape platform or when 60 s had elapsed. A rat was allowed to stay on the platform for 20 s. Then, it was taken from the platform, and the next trial was started. Rats that did not find the platform within 60 s were put on the platform by the experimenter and were allowed to stay there for 20 s. After completion of the 3rd trial, rats were gently dried with a towel, kept warm for an hour and returned to their home cage (Acquisition trial). Twenty-four hours after the last acquisition session (on day 5), a 'probe trial' was used to assess the rat's spatial retention (retention trial) of the location of the hidden platform. During this trial, the platform was removed from the maze and the rat was allowed to search the pool for 60 s before being removed. During this time, animals should spend more time searching the quadrant that previously contained the hidden platform than the other three quadrants.

All tests were conducted between 08:00 A.M. and 12:00 P.M.

2.3. Locomotor activity test

Locomotor activity was measured with a computerised system ($40 \times 40 \times 35$ cm box; May Commat, Ankara, Turkey). The total locomotor activity is expressed as the sum of vertical movement (rearing) and stereotypic activity (grooming). The activity was evaluated for 5 min.

2.4. BDNF immunohistochemistry

Paraffin sections were prepared from rat brains fixed with 10% neutral buffered formalin. Sections were deparaffinised in xylene, rehydrated through a graded alcohol series and washed with PBS. Next, an antigen retrieval procedure was performed by treating the samples in 10 mM citrate buffer (pH 6.0) in a microwave oven twice at 600 W for 5 min each time. The samples were allowed to cool for 20 min at room temperature and incubated in 3% H₂O₂ for 15 min. Sections were then incubated in a blocking serum (Histostain-Plus Kit, Broad Spectrum, Invitrogen, CA, USA) for 10 min at room temperature to block nonspecific binding. The primary rabbit polyclonal anti-BDNF antibody (Santa Cruz-20981) was applied overnight at a 1:100 dilution at room temperature. Negative control samples were prepared by replacing the primary antibody with the appropriate non-immune IgG at the same concentration. After several washes, the slides were incubated with a biotinylated secondary antibody (Histostain-Plus Kit, Broad Spectrum, Invitrogen, CA, USA) for 20 min at room temperature, and diaminobenzidine (DAB) (DAB Substrate Kit, Invitrogen #00-2014) was applied for visualisation. Sections were briefly counterstained with Mayer's haematoxylin (Invitrogen, CA, USA) and mounted with ClearMount (Invitrogen, CA, USA) on glass slides. The slides were examined under a light microscope (Olympus B× 50), and photomicrographs were taken with a Leica DM 100 system (Leica DFC 290HD). All samples were processed and examined as described above. The staining intensity was graded on a semiquantitative scale ranging from no (0), very weak (1+), moderate (2+), strong (3+) and very strong (4+) expression. Percentage of positive cells was defined as follows: 0, <5%; 1, 6–15%; 2, 16–50%; 3, 51–80%; and 4, >80%.

2.5. Drugs and treatment

3-Br 7-NI and L-arginine were from Sigma (St. Louis, USA). 3-Br 7-NI was dissolved in 5% DMSO; however, L-arginine was dissolved in saline. Drugs were freshly prepared and given intraperitoneally (i.p.) in a volume of 0.1 ml per 100 g body weight of rats. Doses of drugs were selected according to behavioural and neurochemical studies to show that the drugs have the intended effects and to confirm the selected doses on locomotor activities; all results were measured (Komsuoglu-Çelikyurt et al., 2011; Utkan et al., 2012).

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