Evidence for the involvement of neuronal nitric oxide synthase and soluble guanylate cyclase on cognitive functions in rats

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A B S T R A C T

Aims: The influence of 3-bromo-7-nitroindazole (3-Br 7-NI), a potent and selective neuronal nitric oxide synthase (nNOS) inhibitor, and [1H-[1,2,4]-oxadiazole[4,3-a]-quinazoline-1-one] (ODQ), a highly selective, irreversible inhibitor of soluble guanylate cyclase (sGC), on working and reference memory and emotional learning was investigated in rats.

Main methods: The effects were assessed in the three-panel runway and step-down passive avoidance task, respectively.

Key findings: 3-Br 7-NI (5, 10, and 20 mg/kg) and ODQ (5, 10, and 20 mg/kg) significantly increased the number of errors and latency of both working and reference memory performance of rats and impaired retention for the passive avoidance task. The effect of 3-Br 7-NI was reversed by L-arginine (250 mg/kg).

Significance: Findings of the study supported the hypothesis that nNOS inhibition disrupts reference and working memory processes in terms of an impairment in the strategies used for solving learning tasks, and, according to these results, nNOS-sGC may be required for emotional learning and both reference and working memory.

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I Introduction

Learning and memory are the most unique mental processes studied in neuroscience. A variety of pharmacological experiments have been performed to understand the underlying mechanisms. The use of nitric oxide synthase (NOS) inhibitors in many different kinds of learning paradigms has shown that nitric oxide (NO) acts as a retrograde messenger in regulating the memory process (Medina and Izquierdo, 1995). 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 NOS. There are three isoforms of NOS: the brain or neuronal form (nNOS), the endothelial form (eNOS) and the inducible form (iNOS). As it is known, both nNOS and eNOS can be expressed in neurons and modulate synaptic plasticity (Barnstable et al., 2004). There is also evidence for sGC in synaptic function by cGMP, which shows that it might be using multiple mechanisms to modulate synaptic efficacy and its actions, including regulation of synaptic plasticity (Barnstable et al., 2004). There is also evidence that eNOS may play an important role in regulating learning and memory processes and nNOS. 3-Bromo-7-nitroindazole (3-Br 7-NI) is relatively more selective inhibitor of nNOS than the other two NOS isoforms and is clearly a more appropriate tool to describe the role of nNOS in the central nervous system (Bland-Ward and Moore, 1995). Therefore, in the current study, we have investigated the effect of a relatively specific nNOS inhibitor 3-Br 7-NI on emotional learning and both reference and working memory processes.

It is well known that soluble guanylate cyclase (sGC), a heterodimeric enzyme that converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP), is a critical component of NO-cGMP signaling pathway. cGMP is a second messenger nucleotide that has been strongly implicated in the process of learning and memory (Prickaerts et al., 2005). There are studies about modulation of synaptic function by cGMP, which shows that it might be using multiple mechanisms to modulate synaptic efficacy and its actions, including regulation of synaptic plasticity (Barnstable et al., 2004). There is also evidence for sGC activation in memory formation in a number of studies (Chien et al., 2005, 2008; Zhuo et al., 1994). Taken together, as a next step, we investigated the effect of 3-Br 7-NI, a potent and relatively selective nNOS inhibitor, and [1H-[1,2,4]-oxadiazole[4,3-a]-quinazoline-1-one] (ODQ), a highly selective, irreversible inhibitor of soluble guanylate cyclase (sGC), on working and reference memory and emotional learning.

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Significance: Findings of the study supported the hypothesis that nNOS inhibition disrupts reference and working memory processes in terms of an impairment in the strategies used for solving learning tasks, and, according to these results, nNOS-sGC may be required for emotional learning and both reference and working memory.
Materials and methods

Animals

Adult male Wistar rats (Kocaeli University, Experimental Medical Research and Application Center, Turkey) weighing 200–300 g were kept in an animal colony at a density of approximately 5–6 per cage for 2 weeks before the experiments. The experiments were conducted between 9:00 a.m. and 12:00 p.m. under standard laboratory conditions, which were maintained (22 ± 2 °C room temperature; 12-h light/dark cycle with lights on at 7:00 p.m.). Tap water and food pellets were provided ad libitum. All animals used in this study were naive to the experimental tests. 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 (6 July 2006, Number 26220). Ethical approval was granted by the Kocaeli University Animal Research Ethics Committee (Kocaeli, Turkey, Project number: HAEK 24).

Drugs

3-Br 7-NI, ODQ and L-arginine HCl were purchased from Sigma-Aldrich (USA), ODQ and 3-Br-7-NI were dissolved in dimethyl sulfoxide (DMSO), whereas L-arginine was dissolved in saline. Behavioral tests were performed 30 min after 3-Br-7-NI (5, 10 and 20 mg/kg) treatment and 20 min after ODQ (5, 10, and 20 mg/kg) treatment. In a separate experiment, L-arginine (250 mg/kg) was injected 20 min prior to NOS inhibitors. All drugs were prepared immediately prior to use 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 behavioral and neurochemical studies to show that the drugs have the intended effects (Fidecka, 2003; Heiberg et al., 2002) and to confirm the selected doses on locomotor activities; all results were measured.

Apparatus and procedures

Passive avoidance learning

In this type of avoidance learning test, the animals were refraining from making the measured response. A step-down variant passive avoidance apparatus was used (Ugo Basile model 7551, Italy). The apparatus (measuring 22 × 21 × 22 cm) consisted of two compartments: a light and dark compartment separated by a guillotine door. On day one (training trial), the rats were placed individually into the light compartment and allowed to explore the boxes to become aware of the environment.

1. Pre-acquisition trial: After 30 s, the door between the two boxes was opened, and the animal moved into the dark compartment freely.
2. The acquisition (training) trial was conducted 15 min after the pre-acquisition trial. Rats were placed in the light compartment, and after a 30 s adaptation period, the door between the compartments was opened. Having completely entered the dark compartment, the door was automatically closed, and an electric foot-shock (0.5 mA) of 3 s duration was delivered to the animal via the grid floor. The time taken to reenter the dark compartment was recorded (training latency). Any animal failing to cross from the light to the dark compartment within 300 s was discarded from the experiment. Animals were then removed from the dark compartment and returned to their home cages. Between each training session, all chamber compartments were cleaned to remove any confounding olfactory cues.

3. Retention trial: Recall of the inhibitory stimulus was evaluated 24 h post-training by returning the animals to the light compartment and recording their latency to enter the dark compartment (four paws in). No foot-shock was applied in this trial. If the animal did not enter the dark compartment within 300 s, it was returned to its cage and a maximum latency of 300 s was recorded. This latency served as a measure of retention performance of step-down avoidance responses (retention latency).

In the present study, rats received 3-Br 7-NI and ODQ 30 min and 20 min before foot-shock training, respectively. Also, L-arginine was injected 20 min before NOS inhibitor.

Three-panel runway test

A three-panel runway test was used to evaluate reference and working memory performances of the rats according to the method described in previous studies by Furuya et al. (1988) and Ohno et al. (1992). The three-panel runway apparatus (175 × 36 × 25 cm, length × width × height) was composed of a start box, a goal box, and four consecutive intervening choice points. Each choice point consisted of a gate with three panels (12 × 25 cm, width × height). Rats were prohibited from passing through two of the three panels in the gate by front stoppers and were also prohibited from returning either to the start box or to a previous choice point by rear stoppers affixed to each of the panels in all gates. When rats reached the goal box, they received food pellets as positive reinforcement (Fig. 1).

At the beginning of the test, all front stoppers were removed so that at each choice point, a rat could pass through any one of the three-panel gates. The rats were forced to repeatedly run the task until the elapsed time from leaving the start box to reaching the goal box consistently fell below 20 s. Once the rats reached this state, they were forced to run the task when the front stopper of only one of the three-panel gates (the correct panel gate) was removed at each choice point.

In the working memory task, six consecutive trials were performed each day at 2 min intervals (one session), and water was freely available between trials in the home cage. The locations of the correct panel gates were held constant within a session but were changed from one session to the next. Thus, 12 different patterns of correct panel gate locations were used in this experiment, as previously described (Furuya et al., 1988; Ohno et al., 1992). In the reference memory task, six consecutive

Fig. 1. Schematic drawing of the three-panel runway apparatus. Rats were allowed to perform the task after the guillotine door was opened in front of the start box. Rats had to pass through four consecutive choice points to obtain food pellets placed in the goal box. Each choice point consisted of a gate with three panels (a, b and c). The rats could pass through only one of the three-panel gates (the correct panel gate) at each choice point.

trials were applied each day at 2-min intervals (one session). The locations of the correct panel gates were both held constant within a session.

The number of times an animal pushed an incorrect panel gate (defined as errors) and the time required for the animal to take food pellets (defined as latency) were recorded for each rat in each trial of a session. The learning criterion was less than 6 errors and less than 12 errors summed across six trials of each session in reference and working memory tasks, respectively. The criterion was defined according to the study reported by Ohno et al. (1992). After the rats achieved this criterion throughout three consecutive sessions, they were divided into groups. The rats that failed to reach the learning criterion were discarded from the study.

**Locomotor activity test**

Locomotor activity was measured with a computerized system (40 × 40 × 35 cm box; May Commat, Ankara, Turkey). The total number of movements was evaluated for a 5-min period.

**Statistical analysis**

The number of errors and latency were summed across all six trials of a session for the reference memory task. They were summed from the second to the sixth trial of a session for the working memory task. They were summed from across all six trials of each session in reference and working memory tests, respectively. The criterion was defined according to the study reported by Ohno et al. (1992). After the rats achieved this criterion throughout three consecutive sessions, they were divided into groups. The rats that failed to reach the learning criterion were discarded from the study.

**Results**

The effect of DMSO:

We used DMSO as a carrier for 3-Br 7-NI and ODQ. DMSO had no effect on either working or reference memory in the three-panel runway tests, emotional learning performance in passive avoidance test or locomotor activity compared to saline control (Fig. 2a, b; Fig. 3a, 3b; Fig. 4).

Effects of 3-Br 7-NI and ODQ on locomotor activity:

Increased locomotor activity may produce behavioral disinhibition and can affect learning and memory processes. To exclude this possibility, the locomotor activity of animals was also assessed by measuring the number of movements over a 5 min period. Statistical analysis of the data showed that 3-Br 7-NI at 5, 10 and 20 mg/kg doses and ODQ at 5, 10 and 20 mg/kg doses does not modify the number of movements in the locomotor activity test (Table 1).

Acquisition process in the three-panel runway apparatus:

The number of errors and latency performance of rats in all 6 trials of a session decreased with repeated training in the reference memory procedure test. After 12–15 training sessions, the rats could run the three-panel runway task within the 6 errors criterion summed across 6 trials.

In the working memory performance test, the number of errors in trial 1 remained stable at approximately 4–5, while the number of...
Effects of ODQ on working memory performance in the three-panel runway test:

Rats received ODQ (5, 10 and 20 mg/kg, i.p.) 20 min before foot-shock training. During the training session (on day 1) of a step-down passive avoidance task, there was no significant difference between all groups (data not shown). However, 3-Br 7-NI (5, 10 and 20 mg/kg, i.p.) treated rats showed a significantly and dose-dependent lower step down latency (SDL) compared to the saline group (F(5,44) = 65.15, p < 0.0001). Decrease in SDL indicated an impairment in learning in the passive avoidance task. This effect of 3-Br 7-NI was reversed by L-arginine (250 mg/kg, i.p.) pretreatment (Fig. 4).

Effects of ODQ on passive avoidance test:

Rats received ODQ (5, 10 and 20 mg/kg, i.p.) 20 min before foot-shock training. During the training session (on day 1) of a step-down type passive avoidance task, there was no significant difference between all groups (data not shown). However, ODQ (5, 10 and 20 mg/kg, i.p.) treated rats showed a significantly and dose-dependent lower SDL compared to the saline group (F(5,44) = 79.09, p < 0.05, Fig. 4). Decrease in SDL indicated an impairment in learning in the passive avoidance task.

Discussion

The main finding of the study was that 3-Br 7-NI and ODQ significantly increase the number of errors and latency performances of rats in the three-panel runway task and impair retention latency in the passive-down avoidance task, which indicates that nNOS inhibition and sGC inhibition impair reference and working memory and play a role in the emotional learning function in rats. It has been reported that NOS inhibitors have an effect on blood pressure; therefore, it is difficult to quantify how and to what extent these effects might have specifically affected cognition (Varner and Beckman, 1994). However, it has been reported that when 3-Br 7-NI is injected systemically, it does not have hypertensive effects at a dose of 20 mg/kg (Rees et al., 1990). Thus, behavioral consequences due to potential 3-Br 7-NI induced hypertensive effects can likely be excluded. Although it is known that i.p. injection of nNOS inhibitors lower baseline cerebral blood flow (Gottho et al., 2001), there is no basic evidence for the effect of 3-Br 7-NI on cerebral blood flow in rats. Furthermore, the animals treated with 3-Br 7-NI and ODQ in our experiments did not alter locomotor activity. DMSO was used as a carrier for both drugs and is in agreement with a previous study; DMSO had no effect on the behavioral performance in any of our experiments. Our results are consistent with previous studies that demonstrated impairment in learning and memory functions of various nitroarginines and 7-NI, a nNOS and eNOS inhibitor (Meyer et al., 1998). An advantage of using 3-Br 7-NI in our study is the fact that 3-Br 7-NI induced hypertensive effects can likely be excluded. Although it is known that i.p. injection of nNOS inhibitors lower baseline cerebral blood flow (Gottho et al., 2001), there is no basic evidence for the effect of 3-Br 7-NI on cerebral blood flow in rats. Furthermore, the animals treated with 3-Br 7-NI and ODQ in our experiments did not alter locomotor activity. DMSO was used as a carrier for both drugs and is in agreement with a previous study; DMSO had no effect on the behavioral performance in any of our experiments. Our results are consistent with previous studies that demonstrated impairment in learning and memory functions of various nitroarginines and 7-NI, a nNOS and eNOS inhibitor (Meyer et al., 1998). An advantage of using 3-Br 7-NI in our study is the fact that it has been described as relatively specific inhibitor of nNOS and limits the confounding inhibition of either eNOS or iNOS.

NOS inhibitors were reported to impair memory in learning-memory tasks, but these findings are discrepant in this context. Some studies reported that NOS inhibitors do not change learning performance in...
the passive avoidance (Böhme et al., 1993; Telegdy and Kokavszyk, 1997) and memory in the Morris water maze (Blokland et al., 1999; Bannerman et al., 1994), whereas other studies reported that they inhibit the retention trial of the passive avoidance (Hölscher and Rose, 1992; Finn et al., 1995; Kopf et al., 2001) and spatial learning in the water maze (Chapman et al., 1992; Toyoda et al., 1996; Prendergast et al., 1998) or the radial arm maze (Zou et al., 1998) and object recognition (Prickaerts et al., 1997). It has been demonstrated that while NO blockade results in an impairment of place-navigation learning, it has no action on sensory and motivational factors (Prendergast et al., 1998; Estall et al., 1993). In this study, the three-panel runway apparatus was used, which is a useful method. Previously, it has been reported that the three-panel runway paradigm distinguishes between the two types of memory because arrangement of the experiment consists of trials. First, reference memory refers to memory for information that remains constant over repeated trials and is therefore trial independent. Second, working memory refers to memory in which the information to be remembered changes in repeated trials, so it is trial-dependent (Olton and Parás, 1979). The choice of this task was thought to be of interest because, to our knowledge, the effect of 3-Br 7-NI and ODQ on cognition was not evaluated by working and reference memory paradigm. Our findings on reference and working memory tasks added new information to the known ability of nNOS inhibitors and sGC inhibitors to counteract memory impairments.

The other test used in this study was the passive avoidance, which is a fear-motivated test and is mainly used in studying learning alterations in rats following drug administration, central nervous system lesions or manipulations.

Long-term potentiation (LTP) is a cellular mechanism for learning and memory functions in the hippocampus (Blackshaw et al., 2003). The predominating NOS isoform in neurons is nNOS, but neurons of the hippocampus and other brain regions additionally contain eNOS (Prast and Philippu, 2001). Although selective nNOS inhibition can inhibit LTP in the striatum and hippocampus, Haul et al. (1999) reported the requirement of eNOS for LTP development. However, Wu et al. (1997) found that 3-Br 7-NI and 1-(2-trifluoromethylphenyl)-imidazole (TRIM) inhibit the induction of LTP. These authors suggested that stimulation of NO production from nNOS is necessary for the induction of LTP in the hippocampus. Similar to pharmacological inhibition, genetic inhibition of nNOS showed impaired spatial performance in the Morris water maze (Weitzdoerfer et al., 2004). Although information on protein expression in the nNOS knockout (KO) mouse brain, in particular in hippocampus, is not fully understood, some proteins that are regulated by NO including nNOS-responsive proteins have been reported. Biochemical derangement therefore may help to interpret or indicate a possible mechanism of specific protein changes in hippocampus of nNOS KO and at least describe the association of abnormal protein expression, impaired cognitive function and the nNOS deficit (Kirchner et al., 2004). nNOS KO mice showed hyperlocomotor activity in a novel environment, increased social interaction in their home cage, decreased depression-related behavior, and impaired spatial memory retention induces abnormal social behavior, hyperactivity and impaired remote spatial memory (Tanda et al., 2009). nNOS signaling also regulates diverse cellular processes during brain development and molecular mechanisms required for higher brain function. nNOS has also been associated with prefrontal cortical functioning, including cognition, of which disturbances are a core feature of schizophrenia. nNOS KO mice also display mild impairments in object recognition memory, showing potential impairments in prefrontal cortex mediated cognitive function (andrea et al., 2011). Such experiments support our present study, in which 3-Br 7-NI was found to impair learning and memory performances of rats. In our study, the reversal of the 3-Br 7-NI impairment effect by L-arginine greatly supports the theory that this effect is specific to NOS. Despite considerable evidence for NO involvement in at least some forms of memory processing (Hölscher and Rose, 1992; Finn et al., 1995; Kopf et al., 2001; Chapman et al., 1992; Toyoda et al., 1996; Prendergast et al., 1998; Zou et al., 1998; Prickaerts et al., 1997), its required function is not known. However, the majority of NO-mediated physiological processes are thought to result from the activation of GC and, in turn, the cGMP activation of protein kinase G (PKG). Edwards et al. (2002) reported that inhibition of GC and PKG impairs retention for the passive avoidance task, suggesting that GC mediates two memory retrieval processes. Interestingly, in a study using sGC inhibitor ODQ and ADP-ribosyltransferase inhibitor nicotinamide, ODQ failed to affect LTP but effectively suppressed cGMP production in the hippocampus; Kleppisch et al. (1999) showed that PKGs are not involved in LTP in mice but that NO induces LTP through an alternative cGMP-independent pathway, possibly ADP-ribosylation. However, there have been studies about the modulation of synaptic function by cGMP, which shows that it may use multiple mechanisms to modulate synaptic efficacy, and its action may include regulating synaptic plasticity (Barnstable et al., 2004). Moreover, it has been shown that, at least in some complex models such as rat cerebellar slices, NO can serve as a chemical signal between neurons such as pyramidal neurons in piriform cortex. NO can permeate the plasma membrane and destroy post-synaptic structures via the generation of reactive oxygen species within the dendrites and cell body. Therefore, NO especially nNOS induction and activation of layer 1 interneurons may be a common denominator linking lesion- and NMDA antagonist-induced death of pyramidal neurons in limbic cortex (Zhou et al., 2007).

It was reported that VC-1 enhances learning behavior in the Morris water maze and passive avoidance (Chien et al., 2005, 2008). In addition, rolipram and sildenafil showed impaired object retrieval performance, demonstrating the cognitive enhancement effect of phosphodiesterase inhibition on a prefrontal task of executive function in monkeys (Rutten et al., 2009). Moreover, a study showed that phosphodiesterase9 inhibition may be a target for treating memory deficits that are associated with aging and neurodegenerative disorders (Van de Staay et al., 2008). There has also been clear evidence that hippocampal cGMP, but not cAMP, is involved in the early stages of object memory consolidation (Prickaerts et al., 2002). Our findings are consistent with these results suggesting that nNOS-sGC-cGMP pathway is involved in the pathophysiology of and memory functions.

Another mechanism of NO signaling is the reaction of NO with cysteine residues in proteins to form nitrothiols and this reversible modification is known as S-nitrosylation (Martiniz-Ruiz and Lamas, 2004). S-nitrosylation is regarded as an important redox signaling mechanism in the regulation of different cellular and physiological functions. However, deregulation of S-nitrosylation has also been linked to human diseases such as neurodegenerative disorders. (Duda et al., 2000; Giasson et al., 2000; Horiguchi et al., 2003). The molecular mechanisms of how nitrosative stress contributes to neurodegenerative disease are not clear, but it is believed that nitration of protein can induce the formation of protein aggregates, which can ultimately cause the degeneration of neurons. Since this study was not designed to explore the effects of nitrosative stress on learning and memory process, we cannot draw any conclusion at the present time.

Conclusion

The current study showed the effect of 3-Br 7-NI in the three-panel runway test for the first time. The findings of the study supported the hypothesis that nNOS inhibition disrupts reference and working memory processes by impairing the strategies used for solving learning tasks and according to these results, nNOS-sGC may be required for emotional learning and both reference and working memory.

Conflict of interest statement

The authors declare no conflict of interest.


