



Effects of 7-nitroindazole, a selective neural nitric oxide synthase inhibitor, on context-shock associative learning in a two-process contextual fear conditioning paradigm



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ABSTRACT

Nitric oxide (NO) is an important retrograde neuronal intracellular messenger which plays an important role in synaptic plasticity and is involved in learning and memory. However, evidence that NO is particularly important for the acquisition of contextual fear conditioning is mixed. Also, little is known about at which stages of the contextual fear conditioning does NO make its contribution. In the present study, we used 7-nitroindazole to temporarily inhibit neural nitric oxide synthase at either the pre-exposure stage or conditioning stage in a two-process paradigm and examined the potential contribution that NO makes to the contextually conditioned fear. Results showed that the expression of contextual fear memory was significantly impaired in rats treated with 7-nitroindazole (30 mg/kg, i.p.) prior to the pairing of context-shock ($p = 0.034$, $n = 8$), but not after the conditioning phase ($p = 0.846$, $n = 8$). In addition, the expression of contextual fear memory and reconsolidation was not significantly impaired by 7-nitroindazole administered prior to the context pre-exposure stage or prior to another context-shock learning. These findings suggest that NO is specifically involved in the acquisition but not the consolidation, retrieval or reconsolidation of contextual fear memory.

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1. Introduction

Nitric oxide (NO) is a soluble, short-lived and freely diffusible gas, which is found in almost all tissues of the body and throughout the central nervous system (Alderton, Cooper, & Knowles, 2001). NO is synthesized from L-arginine by nitric oxide synthase (NOS), which has three isoforms: the endothelial (eNOS), the inducible (iNOS) and the neuronal (nNOS) forms. As an important intracellular messenger in the brain, a growing body of literature suggests that nitric oxide plays an important role in the synaptic plasticity and is important for various forms of learning and memory, especially the hippocampus-dependent one. For instance, spatial learning was found to be impaired in rats by the inhibitor of NOS in a radial-arm maze (Bohme et al., 1993) or Morris water maze (Majlessi, Choopani, Bozorgmehr, & Azizi, 2008); and olfactory memory in a social recognition test (Bohme et al., 1993;

Matus-Amat, Higgins, Barrientos, & Rudy, 2004). In addition, nNOS knockout mice showed an impaired spatial performance in a Morris water maze (Kirchner et al., 2004). One of the mechanisms underlying the NOS inhibitor-induced impairment may be that NO is needed in the induction of long-term potentiation (LTP) (Bohme et al., 1993).

Although it is often claimed that NO activity is related to the acquisition of contextual fear conditioning, a type of hippocampus-dependent learning (Bast, Zhang, & Feldon, 2003; Matus-Amat et al., 2004), existing evidence has been mixed so far. For instance, Kelley et al. reported that nNOS knockout mice showed a severely impaired contextual fear learning compared to wild-type ones (Kelley, Balda, Anderson, & Itzhak, 2009). In line with this study, the selective nNOS inhibitor S-methyl-L-thiocitrulline (SMTC) reduced both short- and long-term memories of contextual (36% inhibition) but not cued fear conditioning. On the other hand, pretraining administration of the NO donor mol-sidine to nNOS knockout mice improved their deficit in short- and long-term memories of contextual fear conditioning (46% increase) (Kelley, Anderson, & Itzhak, 2010). These findings suggest that NO is important for the acquisition of long-term memory of

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contextual fear conditioning. However, others find opposite results. Maren and his colleagues found that systemic administration of 7-nitroindazole, a selective nNOS inhibitor, did not affect the acquisition of contextual fear, a form of learning that depends on both the hippocampus and hippocampal LTP (Maren, 1998), suggesting that NO is not required for the acquisition of contextual fear learning. At this time, it is still not determined whether NO is critical for contextual fear conditioning.

The above mentioned studies used a typical contextual fear conditioning paradigm, that is, an animal is being placed in an apparatus and a few minutes later receives an unsignaled electrical footshock. Subsequently, the animal is tested in the original training context and freezing is recorded as an index of fear memory. In a typical contextual fear conditioning paradigm, the acquisition of a conjunctive contextual representation and context-shock association take place almost simultaneously within a single training session. As such, the effect of NO on the acquisition of a conjunctive contextual representation or context-shock association during contextual fear conditioning may be obscured by the typical training paradigm (Fanselow, 2000, 2010). Moreover, animals in the typical contextual fear conditioning paradigm may be conditioned to the independent features of context instead of a conjunctive representation (Matus-Amat et al., 2004). Consequently, it is possible that conditioning to independent contextual features makes the 7-nitroindazole to fail to inhibit contextual fear conditioning, because nNOS has little effect in cue-induced fear conditioning (Kelley et al., 2010; Maren, 1998).

To address this issue, the present study separates the process of acquisition of a conjunctive contextual representation from the context-shock association process during contextual fear conditioning by employing a two-process training paradigm (Fanselow, 2000, 2010; Matus-Amat et al., 2004). In this paradigm, an animal is first being pre-exposed to a context where it would receive an immediate shock on the next day, then the animal would display substantial freezing in the subsequent test. However, if the animal is not pre-exposed to the context but only receives an immediate shock, almost no freezing would be observed in the subsequent test (Fanselow, 2010). It has been observed that context pre-exposure facilitates the contextual fear conditioning due to that the animal has acquired a conjunctive representation of the context in the pre-exposure stage (Bird & Burgess, 2008). Thus a two-process training paradigm allows us to separate contextual fear conditioning into two processes: (1) pre-exposure to the context so that the animal can acquire a conjunctive representation of the context, and (2) immediate shock in the context so that the animal can form the context-shock associative memory. As such, a two-process paradigm is likely able to help answer the question that which process that NO contributes to in contextual fear conditioning if there is any. Moreover, animals in a two-process paradigm would not condition to contextual features, because shock is immediate and animals do not have the opportunity to encode the features of the shock context (Matus-Amat et al., 2004), which means that the pre-exposed animals must condition to the retrieved conjunctive contextual memory but not to sensory features (Matus-Amat et al., 2004). Thus, the two-process paradigm is a more reliable task to study the effect of 7-nitroindazole on the contextual fear conditioning than a one-process paradigm.

In the present study, we used 7-nitroindazole to temporarily inhibit nNOS prior to the pre-exposure or conditioning phase and examined the roles of NO in the acquisition and expression of contextual fear. Based on our literature review, we hypothesized that NO plays a critical role in the acquisition of conjunctive representation of the context, or/and in the formation of the context-shock associative memory.

2. Materials and methods

Animals and housing. Male Sprague-Dawley rats weighting 200–250 g were purchased from Experiment Animal Center, Chongqing University of Medicine, Chongqing, China. They were initially housed in pairs in transparent cages (47 cm × 32 cm × 21 cm) with corn-cob granule for bedding in a colony on a 12-h light/dark cycle (lighting on at 08:00). The rats had unrestricted access to food and water in their home cages. All animals were handled daily (1 min/day) for 5 days prior to the start of experiments to acclimate them to handling. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals and all procedures were approved by the animal care and use committee at Southwest University, China.

Drugs and choice of doses. 7-Nitroindazole (7-Ni, Sigma-Aldrich Inc., Missouri, USA), a selective inhibitor of nNOS has been reported to prevent spatial learning and hippocampal LTP induction *in vivo* without side effects associated the inhibition of other NOS (MacKenzie et al., 1994). 7-Ni was first dissolved in DMSO and then diluted to the final concentration of 30 mg/ml in 80% soybean oil/20% DMSO. Thus, the final concentration of 7-Ni was 30 mg/ml in a mixed vehicle (soybean oil/DMSO, 80%/20%, v/v). Control animals only received soybean oil/DMSO (80%/20%, v/v). All drugs was administered intraperitoneally (i.p.) in a volume of 1.0 ml/kg at 30 min prior to the start of behavioral tests.

Contexts. There were two contexts (Context A and Context B) used in the present study. Context A was the standard rodent conditioning chambers (30.1 cm × 24.7 cm × 23.3 cm; Clever System Inc., Virginia, USA) with aluminum sidewalls and a Plexiglas rear wall. The floor of each chamber consisted of 18 stainless steel rods (5-mm diameter) spaced 1.6 cm apart (center to center). The rods were wired to a shock source for delivery of the footshock. Background noise (60 dB) was supplied by ventilation fans positioned into the sound-attenuating chests, and yellow lights within the chambers and the fluorescent lights within the experimental room provided illumination. The chambers were cleaned with 70% Ethyl alcohol. Stainless steel pans placed underneath the grid floors were sprayed with a thin film of 70% Ethyl alcohol before the animals were placed inside the boxes. Animals were transported to the experimental rooms in their home cages, which were covered with a black trash bag and arranged on the top of the plastic cart. Context B was the white Plexiglas cages (42.5 cm × 26.2 cm × 5.8 cm) with corn-cob granule housed in another independent experimental room. The fluorescent lights within the experimental room provided illumination. Animals were transported to the Context B in their home cages on the top of the cart and covered with a white trash bag.

Statistical analysis. Freezing data in the test session were statistically analyzed using a factorial repeated measures analysis of variance (ANOVA) with group as the between-subjects factor and test time point as the within-subjects factor. Group differences were further investigated using simple main effect tests (one-way ANOVA) followed by LSD post hoc tests. Difference between groups at the specific test time bin was analyzed using one-way ANOVA, followed by post hoc LSD tests. *T*-test was used to investigate the difference between vehicle and 7-Ni treated groups for the retrieval or reconsolidation of already existing memory trace in Experiment 4. Statistical significance was accepted at $p < 0.05$, two-tailed.

2.1. Experiment 1: Effects of 7-Ni on the acquisition of context-shock associative memory

The objective of Experiment 1 was to determine whether 7-Ni has an effect on the acquisition of context-shock associative memory.

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