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 ʟ-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-ʟ-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 molsidomine 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%). 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 back 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.

Experimental design. Twenty-four rats were randomly assigned to the vehicle, 7-Ni or pseudo-exposure groups. The two-process training paradigm of contextual fear conditioning was composed of two training phases in two days as previously described (Chang & Liang, 2012). The experimental design is illustrated in Fig. 1A. Briefly, in the pre-exposure session on the first day, rats were carried to Context A for pre-exposure of 10 min except for animals in the pseudo-exposure group which were exposed to Context B for the same duration. After exposure, the animals were returned to the vivarium.

In the context-shock session on the second day, thirty min prior to training, 7-Ni treated rats received 7-Ni (30 mg/kg) in their homecages ($n = 8$), whereas the vehicle ($n = 8$), pseudo-exposure ($n = 8$) rats received soybean oil/DMSO (80%/20%, v/v, 1.0 ml/kg). All rats were then transported to Context A and received an unsignaled footshock (2 s, 0.9 mA) 15 s after being placed into conditioning chambers. Rats were removed from the Context A immediately after the shock and then returned to the vivarium. According to the previous work, 15 s of re-exposure was sufficient to retrieve the pre-existing contextual memory of pre-exposure, but not enough for the animals without pre-exposure to Context A to encode a new contextual representation (Burman, Murawski, Schiffino, Rosen, & Stanton, 2009; Chang & Liang, 2012). Thus only the pre-existing context representation was supposed to be associated to shock in this session.

Twenty-four hours later, all rats were transported back to the Context A for a 6-min retention test. Behavior was recorded using a video tracking equipment and analyzed with a computer software (FreezingScan, Clever System Inc., Virginia, USA). Freezing behavior, an index of conditional fear in rat, was defined as the absence of any visible movement for longer than 1 s except for respiration.

2.2. Experiment 2: Effects of 7-Ni on the consolidation of context-shock associative memory

In Experiment 1, we explored the role of NO on the acquisition of the context-shock associative memory. The objective of Experiment 2 was to determine whether 7-Ni has an effect on the consolidation of context-shock associative memory. In this experiment, rats were given 7-Ni (30 mg/kg) or vehicle (soybean oil/DMSO) immediately after contextual fear conditioning training.

Experimental design. Twenty-four rats were randomly assigned to the vehicle, 7-Ni or pseudo-exposure groups ($n = 8$/group). The two-process training paradigm was used as described in Experiment 1 with some modifications (Fig. 2A). Briefly, in the pre-exposure session on the first day, rats were carried to Context A for pre-exposure of 10 min except for those in the pseudo-exposure group which were exposed to Context B for the same duration. After that, the rats were returned to the vivarium. Twenty-four hours after training, all rats were then transported to Context A and received an unsignaled footshock (2 s, 0.9 mA) 15 s after being placed into conditioning chambers. Rats were removed from Context A immediately after shock and then, they either received an injection of 7-Ni (30 mg/kg) or vehicle. Rats in the pseudo-exposure group received an injection of soybean oil/DMSO (80%/20%, v/v, 1.0 ml/kg). After that, they were returned to the vivarium. In the test session on the second day, all rats were transported back to Context A for a 6-min retention test as described in Experiment 1.

2.3. Experiment 3: Effects of 7-Ni on the formation of a conjunctive contextual representation

The objective of Experiment 3 was to determine whether 7-Ni has an effect on the process of acquiring the contextual
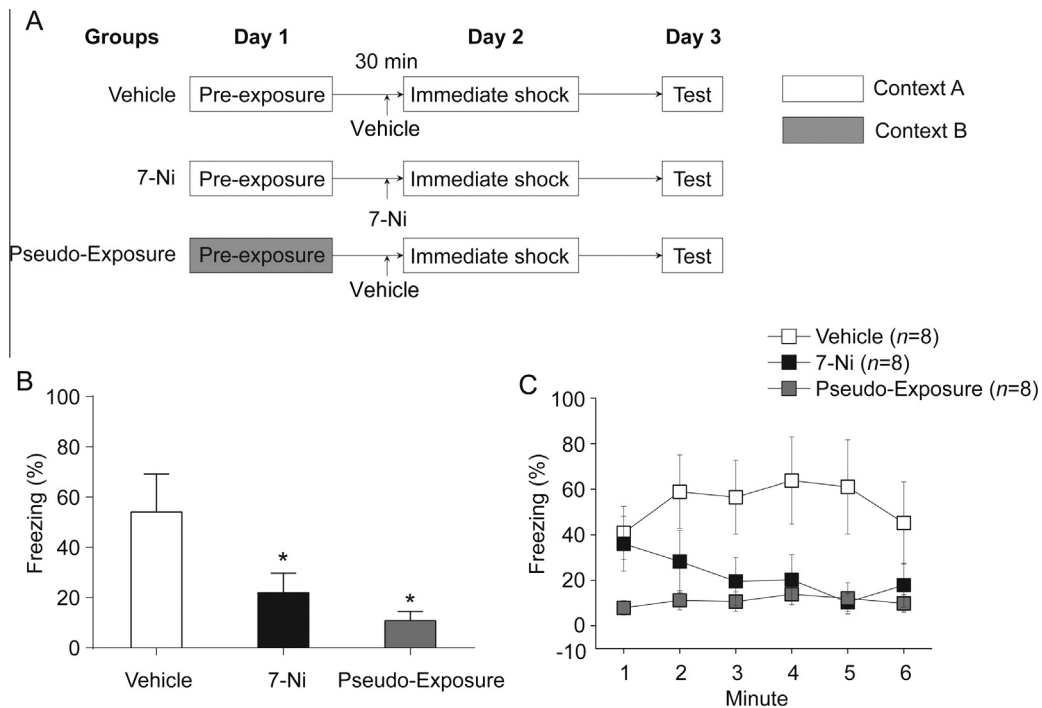

Fig. 1. Effects of 7-Ni on the acquisition of context-shock associative fear memory. (A) A schematic illustration of the experimental procedure. (B) The overall average of percentage time spent on freezing for each group during the retention test. Contextual freezing was significantly impaired in rats treated with 7-Ni (30 mg/kg) prior to contextual fear conditioning ($p = 0.034$). Rats in pseudo-exposure group froze significantly less than the vehicle control group ($p = 0.006$). (C) Freezing behavior (percentage time) was scored for each minute during the 6-min testing session. There is no group × minute interaction among groups [$F(10, 105) = 1.366$, $p = 0.206$]. * $p < 0.05$, significant difference compared to vehicle group using one-way ANOVA.
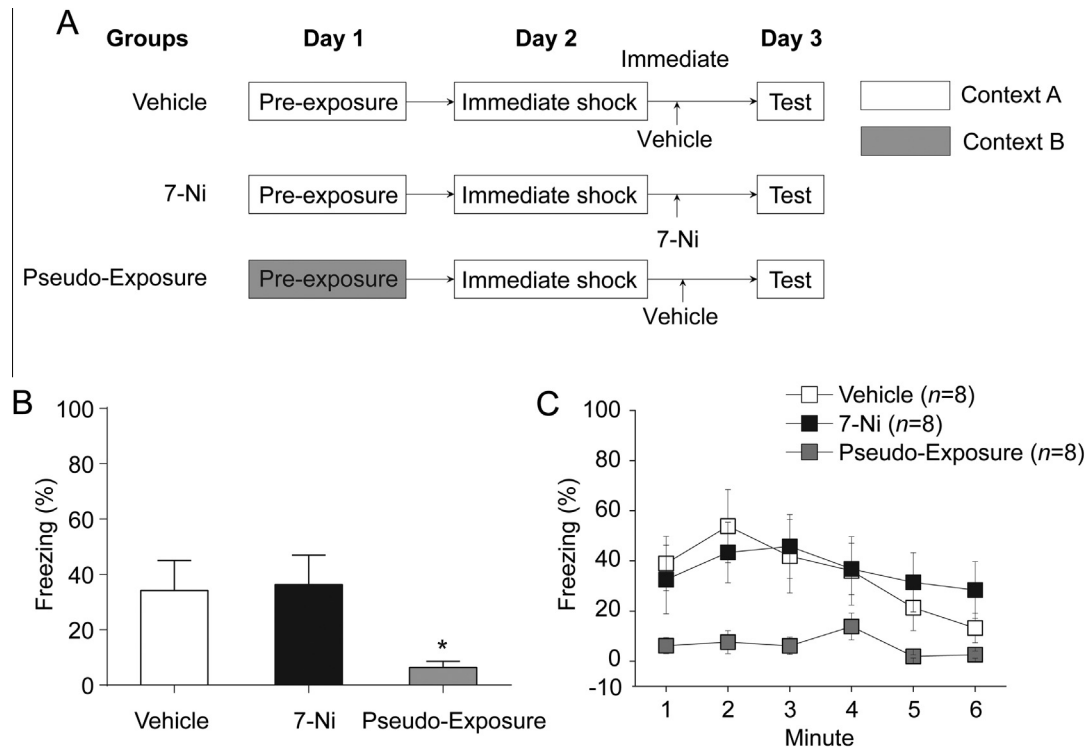
Fig. 2. Effects of 7-Ni on the consolidation of context-shock associative fear memory. (A) A schematic illustration of the experimental procedure. (B) The overall average of percentage time spent on freezing for each group during the retention test. Contextual freezing was not significantly impaired by 7-Ni immediately after contextual fear conditioning ($p = 0.846$). Rats in pseudo-exposure group froze significantly less than the vehicle control group ($p = 0.037$). (C) Freezing behavior (percentage time) was scored for each minute during the 6-min testing session and freezing in one minute was combined into a bin. There is no group × minute interaction among groups [$F(10, 105) = 3.364$, $p = 0.054$]. * $p < 0.05$, significant difference compared to vehicle group using one-way ANOVA.

representation, as there is no evidence supporting a role of NO in the acquisition of the conjunctive contextual representation. Rats were given 7-Ni (30 mg/kg) or vehicle (soybean oil/DMSO) 30 min before pre-exposure session in a two-process paradigm.

Experimental design. Twenty-four rats were randomly assigned to vehicle, 7-Ni or pseudo-exposure groups ($n = 8$/group). The two-process training paradigm was used as previously described in Experiment 1 with some modifications (Fig. 3A). Briefly, in the pre-exposure session on the first day, thirty min prior to pre-exposure, the 7-Ni treated rats were given an injection of 7-Ni (30 mg/kg) in their homecages and the vehicle ones received soybean oil/DMSO (80%/20%, v/v, 1.0 ml/kg). The pseudo-exposure rats also received an injection of soybean oil/DMSO (80%/20%, v/v, 1.0 ml/kg). Thirty min after administration, rats were exposure to Context A for 10 min except for animals in the pseudo-exposure group which were exposed to Context B for the same duration. The rest of the procedure was identical to that of Experiment 1.

2.4. Experiment 4: Effects of 7-Ni on the retrieval and reconsolidation of pre-existing contextual fear memory

The objective of Experiment 4 was to determine whether 7-Ni blocks the retrieval and reconsolidation of contextual memory during the reconsolidation phase which may update already existing contextual fear during a secondary learning 24 h after the first contextual fear conditioning. To rule out the possibility that NO inhibition prevents context-shock association by disrupting updating the contextual memory with shock, we used a tested reconsolidation paradigm in which animals were first trained to a single-shock association and then on the next day NO signaling was blocked with 7-Ni prior to the second context-shock learning. The effects of 7-Ni on updating of contextual memory was tested on the third day.

Experimental design. Sixteen rats were randomly assigned to vehicle or 7-Ni groups. The reconsolidation paradigm was composed of two training phases in two days as previously described. The experimental design is illustrated in Fig. 4A. Briefly, on the first day, 3 min after being placed in Context A, the rats received an unsignaled footshock (0.9 mA, 2 s). Sixty seconds after the shock, animals were transported to their vivarium. On the second day, the 7-Ni treated rats were given an injection of 7-Ni (30 mg/kg) in their home cages and the vehicle ones received soybean oil/DMSO (80%/20%, v/v, 1.0 ml/kg) 30 min before an identical training trial, and then they were tested in the Context A for 6 min 24 h later as Experiment 1.

3. Results

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

Fig. 1B shows the overall average of percentage time spent on freezing for each group during the retention test. One-way ANOVA and post hoc test on freezing showed that contextual freezing was significantly impaired in rats treated with 7-Ni (30 mg/kg) prior to contextual fear conditioning ($F(2,21) = 5.043$, $p = 0.034$). Additionally, the rats in pseudo-exposure group froze significantly less than the vehicle control ones ($p = 0.006$), confirming that the animals could not form the context-shock associative memory after an immediate shock if they had no chance to obtain a conjunctive contextual representation before conditioning (Burman et al., 2009). Fig. 1C shows freezing was scored in the test session and blocked into 6 bins of each minute. Freezing was normalized to 60 s and averaged within each bin. A 3 (Group) × 6 (minute) repeated measures ANOVA revealed a significant main effect of group [$F(2,21) = 5.043$, $p = 0.016$], but no significant test time
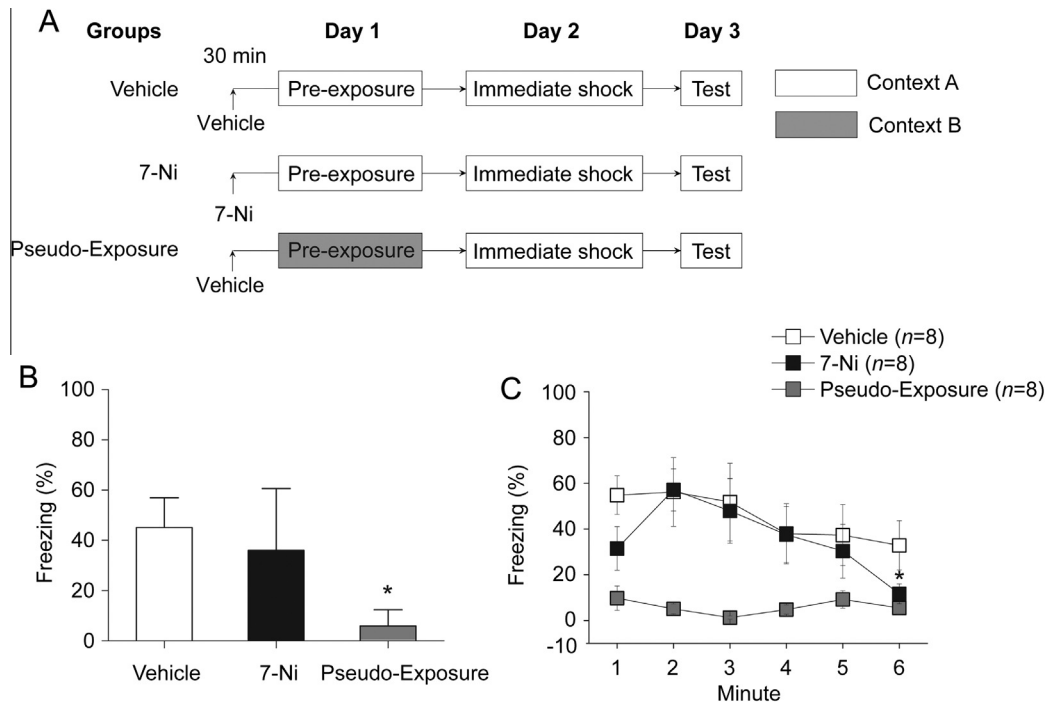
Fig. 3. Effects of 7-Ni on the formation of a conjunctive contextual representation. (A) A schematic illustration of the experimental procedure. (B) The overall average of percentage freezing time for each group during the retention test resulted from the inhibition of nNOS during the formation of a conjunctive context representation. Contextual freezing was not significantly impaired by 7-Ni prior to the pre-exposure ($p = 0.459$). (C) Freezing behavior (percentage time) was scored for each minute during the 6-min testing session. There is a significant group × minute interaction among groups [$F(10, 105) = 2.776$, $p = 0.004$]. Rats injected with 7-Ni froze less than vehicle-treated ones only at the sixth minute in the testing session ($p = 0.043$), which suggests that NO may be involved to some extent in the formation of a conjunctive contextual representation. $^*p < 0.05$, significant difference compared to vehicle group using one-way ANOVA.
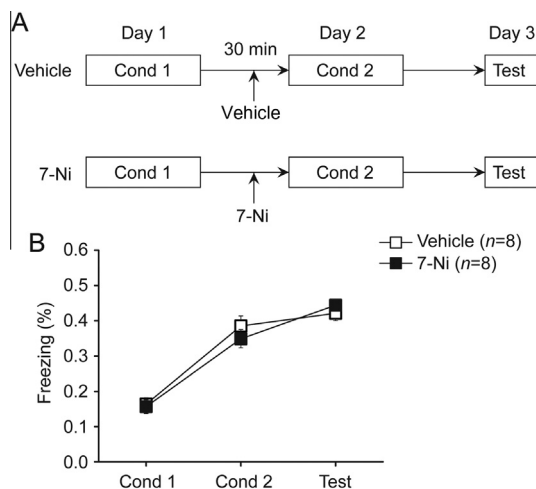

Fig. 4. Effects of 7-Ni on the retrieval and reconsolidation of already existing memory trace. (A) A schematic illustration of the experimental procedure. (B) Freezing behavior (percentage time) was scored during the period before footshock presentation (Condition 2) and 6-min testing session. Rats received 30 mg/kg 7-Ni 30 min prior to Condition 2 did not differ from the vehicle controls before footshock presentation ($p = 0.366$) and in the subsequent test ($p = 0.379$).

[$F(5, 105) = 0.641$, $p = 0.366$] or group × minute interaction [$F(10, 105) = 1.366$, $p = 0.206$].

3.2. Experiment 2: Effects of 7-Ni on the consolidation of context-shock associative memory

Fig. 2B shows the overall average of percentage time spent on freezing for each group during the retention test. One-way ANOVA and post hoc test on freezing revealed that contextual freezing was

not significantly impaired by 7-Ni immediately after contextual fear conditioning ($F(2, 21) = 3.590$, $p = 0.846$), suggesting that inhibition of nNO during the consolidation period does not affect the consolidation of context-shock associative memory. Additionally, rats in pseudo-exposure group froze significantly less than the vehicle controls ($p = 0.037$). Fig. 2C shows freezing was scored in the test session and blocked into 6 bins of each minute. Freezing was normalized to 60 s and averaged within each bin. A 3 (Group) × 6 (minute) repeated measures ANOVA revealed a significant main effect of group [$F(2, 21) = 3.590$, $p = 0.046$], and test time [$F(5, 105) = 9.992$, $p = 0.005$], but no group × minute interaction [$F(10, 105) = 3.364$, $p = 0.054$].

3.3. Experiment 3: Effects of 7-Ni on the formation of a conjunctive contextual representation

Fig. 3B shows the overall average of percentage freezing time for each group during the retention test resulted from the inhibition of nNOS during the formation of the conjunctive context representations. One-way ANOVA and post hoc test on freezing showed that contextual freezing was not significantly impaired by 7-Ni prior to the pre-exposure ($F(2, 21) = 5.741$, $p = 0.459$), suggesting that NO was not involved in the formation of the contextual representation. Additionally, rats in the pseudo-exposure group froze significantly less than the vehicle controls ($p = 0.004$). However, a 3 (Group) × 6 (minute) repeated measures ANOVA showed a significant main effect of group [$F(2, 21) = 5.741$, $p = 0.010$], test time [$F(5, 105) = 5.816$, $p = 0.000$], and group × minute interaction [$F(10, 105) = 2.776$, $p = 0.004$]. To identify the time point(s) at which 7-Ni treated rats differed from the vehicle-treated ones, one-way ANOVAs followed by post hoc tests were used. Results showed that rats injected with 7-Ni froze less than vehicle-treated ones only at the sixth minute in the testing session

($p = 0.043$), indicating that NO only has a very weak effect on the formation of conjunctive contextual representations (Fig. 3C).

3.4. Experiment 4: Effects of 7-Ni on the retrieval and reconsolidation of pre-existing contextual fear memory

Fig. 4B shows the effects of 7-Ni on the retrieval and reconsolidation of the already existing memory trace. *T*-test showed that no significant difference between the vehicle and 7-Ni treated groups in the period before the footshock presentation during Condition 2 ($p = 0.366$, $n = 8$), indicating that 7-Ni administration did not interfere with the retrieval of pre-existing memory trace. In the retention test on the third day, contextual freezing was also not different between the groups ($p = 0.379$, $n = 8$), suggesting that NO is not involved in the reconsolidation of already existing memory trace.

4. Discussions

Previous studies investigated the roles of NO in the contextual fear conditioning, but none paid enough attention to the question of at which stage does NO contribute to the formation of the contextual fear memory. In the present study, we determined that NO is specifically involved in the acquisition of context-shock associative memory instead of the formation of a conjunctive contextual representation or retrieval/reconsolidation of pre-existing contextual fear memory.

As mentioned in the Introduction, previous studies have reported that NO is involved in contextual fear learning and memory. For example, Kelly et al. show that contextual fear learning was severely impaired in nNOS KO mice compared with WT counterparts but the cued fear learning was only slightly impaired in nNOS KO mice (Kelley et al., 2009, 2010). They also show that pre-training administration of selective nNOS inhibitor S-methyl-L-thiocitrulline to WT mice impaired both short- and long-term memories of conditioned contextual but not cued fear (Kelley et al., 2010). In addition, pretraining administration of NO donor molsidomine to nNOS KO mice improved their deficits in short- and long-term contextual fear memory, indicating that neuronal NO is important for contextual fear learning (Kelley et al., 2010). However, Maren and his colleagues revealed that systemic administration of 7-Ni did not affect the acquisition of contextual fear (Maren, 1998). In the present study, we used a two-process contextual fear conditioning paradigm and revealed that 7-Ni did inhibit contextual fear conditioning. Besides the differences in the specific paradigm used, one other factor that may contribute to this discrepancy may be the "ceiling effect" that animals in Maren's study displayed as they were shocked multiple times. Rats in our study were shocked only once and they did not show a maximal level of freezing. This explanation was confirmed by a recent study in which nNOS KO mice that received multiple trainings also overcame deficits in contextual fear learning (Kelley et al., 2009).

In a two-process contextual fear conditioning paradigm, animals are first pre-exposed to a context, and then re-exposed to the same context followed a footshock. Thus, the pre-existing contextual memory will be retrieved and reconsolidated when animals are re-exposed to the same context on the second day, which enables updating of a previously acquired memory through additional learning. As such, it is necessary to rule out a possibility that NO inhibition prevents context-shock association by disrupting the retrieval and/or reconsolidation of pre-existing contextual memory. In Experiment 4, we examined this possibility and found that 7-Ni administration did not interfere with the retrieval of already existing memory trace and NO is not involved in the reconsolidation of existing memory trace. Therefore, we can conclude that

inhibition of nNOS interferes with the association of context-shock via disrupting context-shock associative learning instead of preventing the retrieval or reconsolidation of pre-existing contextual memory.

In this study, we determined the specific processes (acquisition, consolidation, retrieval or reconsolidation of fear memory or the formation of contextual representation, etc.) in which NO contributes to contextual fear learning and memory. We dissociated the contextual fear conditioning temporally from the acquisition of a conjunctive contextual representation and the formation of context-shock associative memory in a two-process contextual fear conditioning paradigm and revealed that it is the acquisition of context-shock associative memory that is dependent on NO. One-way ANOVA showed that overall freezing time during the retention test was not impaired by the inhibition of nNOS during the formation of the conjunctive context representation, and freezing only at the sixth minute in the testing session was slightly reduced ($p = 0.043$), which suggests that NO is not likely involved in the formation of conjunctive contextual representation. This issue needs to be examined further in the future research. Furthermore, our data showed that inhibition of nNOS during the consolidation period did not reduce contextual freezing in the subsequent retention test (Fig. 2), which is consistent with previous studies that inhibition of nNOS during the consolidation period does not disrupt contextual fear condition in a typical contextual fear conditioning paradigm (Kelley et al., 2010).

The underlying mechanisms that NO contributes to the formation of context-shock memory maybe involve the synaptic plasticity in the hippocampus, medial prefrontal cortex (mPFC) and amygdala. The dorsal and ventral hippocampus have been shown to be involved in the acquisition of context-shock associative memory (Matus-Amat et al., 2004; Rudy & Matus-Amat, 2005), and NO signaling plays a critical role in the mechanisms of the hippocampus-dependent learning and synaptic plasticity (Bohme et al., 1993) (Majlessi et al., 2008) (Matus-Amat et al., 2004) (Kirchner et al., 2004). Thus NO signaling in the dorsal and ventral hippocampus may be related to contextual fear learning. In addition, rats given intra-lateral amygdala infusions of either the PKG inhibitor or activator showed impaired or enhanced Pavlovian fear conditioning respectively, suggesting NO-cGMP-PKG signaling may promote synaptic plasticity and fear memory formation in the lateral amygdala (Ota, Pierre, Ploski, Queen, & Schafe, 2008). Therefore, NO signaling in the amygdala may be also involved in the formation of context-shock associative memory. Furthermore, it was reported that the mPFC has been implicated in the acquisition of contextual fear conditioning (Asok, Schreiber, Jablonski, Rosen, & Stanton, 2013; Gilmartin & Helmstetter, 2010; Stern, Gazarini, Vanvossen, Hames, & Bertoglio, 2014). However, it is not clear about the role of NO in mPFC for the cue-induced or contextual fear conditioning. As such, the future study should focus on the role of NO signaling in the hippocampus, amygdala and mPFC for context-shock associative learning.

Although many studies investigated the role of NO signaling in the spatial memory and navigation, the present study was the first that explored the role of NO in the formation of conjunctive contextual representations. A typical contextual fear conditioning paradigm involves one-process and is used to study contextual learning and memory. However, this paradigm mixes the acquisition of a conjunctive contextual representation or context-shock association within a single contextual fear conditioning session (Fanselow, 2000, 2010). In this regards, thus a two-process contextual fear conditioning paradigm is a more appropriate paradigm to study contextual learning and memory. In a two-process contextual fear conditioning paradigm, the inhibition of nNOS before contextual pre-exposure did not disrupt context-shock conditioning on the next day, but the animals had not pre-exposed to condition-

ing context did not develop contextual fear, suggesting that animals administered 7-Ni acquired a contextual memory and formed a conjunctive contextual representation. Although contextual learning and spatial learning both depends on the hippocampus (Bird & Burgess, 2008; Majlessi et al., 2008), the involvement of NO in spatial learning does not mean NO must be involved in contextual learning. The similar discrepancy was also observed in other studies, for example, a short-term treatment of UE2316, a selective 11β-hydroxysteroid dehydrogenase type 1 inhibitor, improved spatial memory in mice, but impaired contextual fear memory (Wheelan et al., 2015). In addition, inhibition of adult hippocampal neurogenesis disrupts contextual learning but spares spatial working memory (Hernandez-Rabaza et al., 2009).

5. Conclusions

Taken together, the present study determined that NO is required for contextual fear memory conditioning, and revealed that NO contributes to the acquisition but not consolidation of context-shock associative memory. Whereas, there is no enough evidence to determine whether NO is involved in the formation of the conjunctive contextual representation. This issue needs to be further examined.

Conflict of interest

The authors have no conflict of interest.

Acknowledgement

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