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Activation of the Noergic System of the Nucleus Accumbens on Presentation of Contextual Danger Signals

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Studies in Sprague–Dawley rats using intracerebral microdialysis combined with HPLC showed that presentation of the animals with a chamber in which they had previously acquired a conditioned reflex fear reaction (combination of a tone and electric shocks) led to increases in extracellular citrulline (a co-product of NO synthesis) in the medial segment of the nucleus accumbens. This increase was prevented by local administration of the NO synthase inhibitor 7-nitroindazole (0.5 mM). The increase was significantly smaller in amplitude than the increase in the citrulline level induced by combined presentation of the tone and the chamber but was no different from changes in citrulline levels seen during this test in the lateral segment of the nucleus accumbens. These data provide evidence that contextual danger signals activate neuronal NO synthase in the medial and perhaps the lateral segments of the nucleus accumbens, leading to increases in extracellular citrulline and, probably, increased NO production in this part of the brain.

KEY WORDS: intracerebral microdialysis, nitric oxide, nucleus accumbens, conditioned reflex fear reactions.

Contexts in which bright, emotionally colored events occur can subsequently operate as trigger stimuli initiating emotional reactions, becoming the decisive factor in selecting behavioral programs [4, 12]. An experimental model for this type of emotional memory is provided by conditioned reflex fear reactions to contextual stimuli arising on presentation of the chamber in which animals have previously been subjected to unavoidable electrocutaneous stimulation [5]. This conditioned reflex reaction often forms concurrently with the acquisition of conditioned reflex fear reactions to "classical" conditioned stimuli (tone + electric shocks delivered via the floor) and can contribute to the neurochemical and behavioral measures of the latter [13]. Published data provide evidence that the nucleus accumbens is among the brain structures involved in the formation and execution of conditioned reflex fear reactions to classical and contextual stimuli [5, 9, 10, 14], this being an area of the ventral striatum with an important role in controlling motivational and emotional processes of different modalities [11]. In particular, we have recently demonstrated that presentation of animals in a conditioned reflex chamber with a tone previously combined with electric shocks in this same chamber leads to increases in extracellular citrulline levels, this being a co-product of nitric oxide (NO) synthesis, in the medial segment of the nucleus accumbens [2], which is blocked by administration of NO synthase inhibitors into this structure [3]. These data represent the first demonstration that sound and contextual danger stimuli presented simultaneously evoke activation of NO synthase in the medial segment of the nucleus accumbens. We suggest that these conditioned reflex rearrangements may, albeit partially, be induced by the contextual stimuli associated with pain reinforcement. The present study addresses verification of this hypothesis. Its aims were to investigate changes in the levels of extracellular citrulline (a co-product of NO synthesis) in the medial segment of the nucleus accumbens on presentation of animals with a chamber in which they had previously (during acquisition of a conditioned reflex fear reaction) received unavoidable electrocutaneous stimulation, and to investigate the relationship between these changes and blockade of neu-

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ronal NO synthase in this part of the brain. A further aim was to study the specificity of these changes in citrulline levels for the medial segment of the nucleus accumbens. This was addressed by determining whether extracellular citrulline levels in the lateral segment of the nucleus accumbens change during the execution of the conditioned reflex fear reaction to contextual stimuli, as this part of the nucleus accumbens is indicated by published data [9, 10] to be activated during this type of conditioned reflex behavior.

METHODS

Studies were performed using 52 male Sprague–Dawley rats weighing 260–330 g. Anesthetized (Rometar, 1.4 μg/100 g, and Zoletil, 5 mg/100 g, i.m.) animals underwent implantation of dialysis catheters into the medial (n = 44) and lateral (n = 8) segments of the right nucleus accumbens, as described previously [1–3, 15]. Microdialysis experiments were performed on the second post-implantation day. At the beginning of the experiments, rats were placed in daytime home cages and dialysis perfusion of the nucleus accumbens with artificial cerebrospinal fluid was started [15]. Animals with dialysis cannulae in the medial segment of the nucleus accumbens were divided into three experimental groups (experimental group 1, n = 15; experimental group 2, n = 8; experimental group 3 (n = 6) and two control groups (control group 1, n = 7; control group 2, n = 8). Rats with cannulae in the lateral segment of the nucleus accumbens constituted a further experimental group (experimental group 4, n = 8). A conditioned reflex fear reaction was developed in animals of all experimental groups 90 min after the beginning of the experiment, as described previously [2, 3]. Each rat was placed in the conditioned reflex chamber for 5 min, where it was presented with the conditioned stimulus (a tone of 1000 Hz for 10 sec) combined during the last second with electrocutaneous stimulation of the paw (0.5 mA, 1 sec). The rat was then returned to the daytime home cage. Training sessions were repeated after an interval of 1 h. The same procedure was followed with animals of control group 1, but without pain stimulation. Rats of control group 2 were placed in the conditioned reflex chamber for 5 min on two occasions, separated by 1 h, where they were presented with the same sound and pain stimuli (each stimulus was presented five times) non-simultaneously and in random order. During this control test, the only conditioned signal associated with the shocks was the chamber, as the sound, although presented, never coincided with the shocks. Baseline portions of dialysate (six samples, each of 5 min) were collected from animals of all groups after 25 min. Rats of experimental groups 1 and 4 and control groups 1 and 2 were then placed in the conditioned reflex chamber for 10 min, after which they were returned to their daytime home cages. After collection of baseline portions of dialysate, animals of experimental group 2 were also placed in the conditioned reflex chamber for 10 min, where they were presented with a tone (1000 Hz, 10 sec) each minute. Animals were then returned to their daytime home cages. In animals of experimental group 3, the perfusion solution was changed to solution containing a neuronal NO synthase inhibitor (7-nitroindazole, 7-NI, 0.5 mM, MB Biochemicals, USA) in artificial cerebrospinal fluid 25 min after dialysate collection started. After a further 30 min, they were placed in the conditioned reflex chamber for 10 min and then returned to their daytime home cages. Dialysate collection (5-min portions) in all groups was completed 20 min after return of rats to their daytime home cages. Citrulline levels were measured by HPLC with electrochemical detection [1]. A chromatography system as described previously [15] was used. Dialysate citrulline contents were expressed as percentages of individual mean pre-test values. Morphological monitoring of cannula positioning in the nucleus accumbens was performed when experiments were complete. Rats with cannulae located in the medial and lateral segments of the nucleus accumbens (depending on group) were included in the analysis (Fig. 1). Statistical analysis was performed using SigmaStat (3.0). changes in citrulline levels during behavioral tests and pharmacological treatments were compared with baseline values by unifactorial ANOVA. If this analysis revealed significant changes it was followed by comparison of changes at each time point with baseline levels using Student's t test. Intergroup comparisons were performed by two-factor analysis of variance followed by comparison of groups at each time point using Student's t test.

RESULTS

Baseline dialysate citrulline levels in the medial and lateral segment of the nucleus accumbens in these experiments were 31 ± 4 nM (n = 44) and 30 ± 7 nM (n = 8), respectively.

Presentation of animals of experimental group 1 (n = 15) with the chamber in which they had previously acquired the conditioned reflex fear reaction (combination of tone with shocks) led to a small but long-lasting (20 min) increase in the extracellular citrulline level in the medial segment of the nucleus accumbens compared with individual baseline values (Fig. 2, A; $F_{(11,154)} = 9.2$, $p < 0.001$) with a peak (131 ± 6%; $t = 10.7$, $p < 0.001$) after the animals were returned to their home cages. This increase was also significant, as demonstrated by two-factor analysis of variance, as compared with the extracellular citrulline level in the medial segment of the nucleus accumbens of animals of control group 1 (n = 7) on presentation of the chamber without shocks (Fig. 2, A; $F_{(11,240)} = 2.7$, $p = 0.003$). During this test, the citrulline level in animals of control group 1 showed no significant change from the individual pre-test baseline value (Fig. 2, A; $F_{(11,66)} = 1.1$, $p = 0.4$).

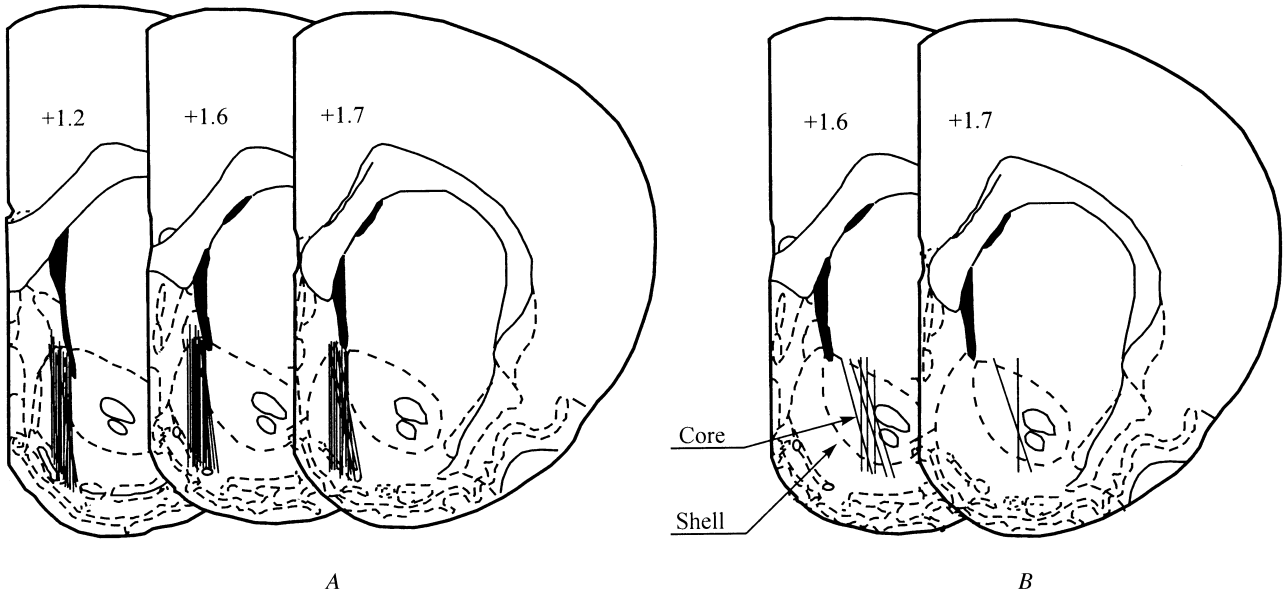

Fig. 1. Positioning of dialysis cannulae in the medial (*A*) and lateral (*B*) segments of the nucleus accumbens. Numbers show distances (mm) from the bregma. "Shell" and "core" are the segments of the nucleus accumbens.

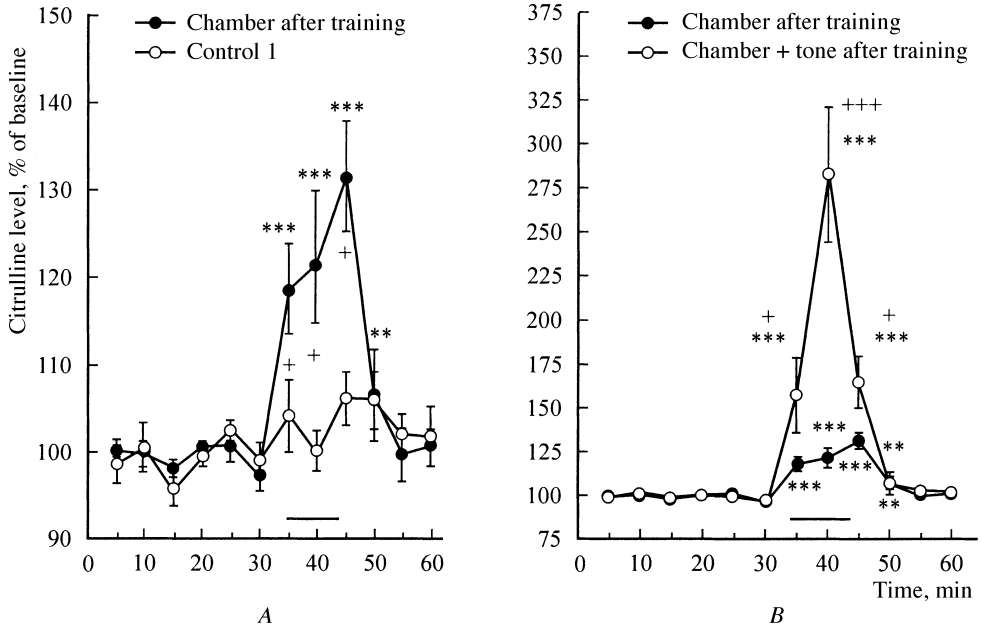

Fig. 2. Changes in extracellular citrulline levels in the medial segment of the nucleus accumbens. *A*) On presentation of animals with the chamber in which they had previously been trained (Chamber after training) and control animals (Control); *B*) on presentation of trained animals with the chamber plus the tone (Chamber + tone after training) or the chamber (Chamber after training). The *X* axis shows time, min; the *Y* axis shows citrulline levels, % of baseline. Bars on plots show errors of the mean. The horizontal lines show the period spent in the chamber. Significant differences on comparison with baseline: $^{*}p < 0.01$; $^{**}p < 0.001$. Significant differences between groups: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$.

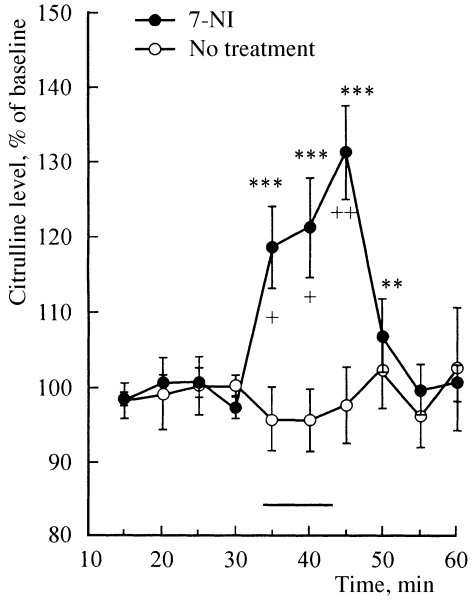

Fig. 3. Changes in extracellular citrulline levels in the medial segment of the nucleus accumbens on presentation of the chamber in which training had taken place in rats given and not given 7-nitroindazole (7-NI) into this structure. For further details see caption to Fig. 2.

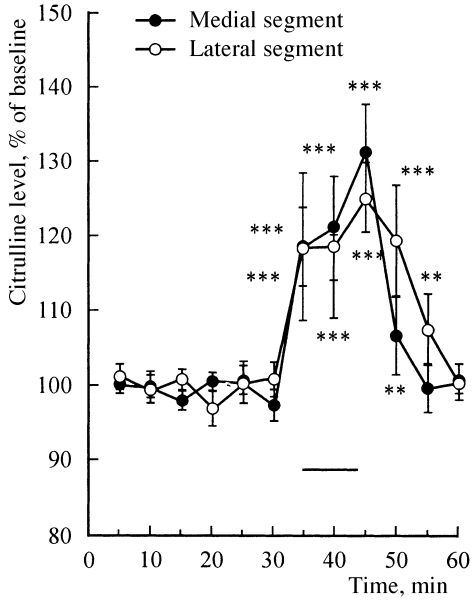

Fig. 4. Changes in extracellular citrulline levels in the medial (Medial segment) and lateral (Lateral segment) segments of the nucleus accumbens on presentation of the chamber in which training had previously taken place. For further details see caption to Fig. 2.

Presentation of animals of experimental group 2 ($n = 8$) with the tone and chamber which had previously been combined with shocks was accompanied by significant (maximum 284 ± 40%; $t = 11.8$, $p < 0.001$) increases in extracellular citrulline in the medial segment of the nucleus accumbens compared with pre-test baseline (Fig. 2, *B*; $F_{(11,77)} = 15.5$, $p < 0.001$), which is consistent with our previous data [2, 3]. Intergroup comparison showed that this increase was greater than the increase in citrulline levels in animals of experimental group 1 during presentation of the chamber (Fig. 2, *B*; $F_{(11,252)} = 17.6$, $p < 0.001$).

Administration of the neuronal NO synthase inhibitor 7-nitroindazole (0.5 mM) into the medial segment of the nucleus accumbens in animals of experimental group 3 ($n = 6$) had no long-lasting effect on baseline citrulline levels, which is consistent with our previous results [3]. A small increase in this parameter (120 ± 10%) occurred in the first five minutes of administration ($F_{(9,45)} = 3$, $p = 0.01$), after which the citrulline level returned to baseline.

Presentation of animals of experimental group 3 (given 7-nitroimidazole) with the chamber in which they had previously acquired the conditioned reflex fear reaction (combination of tone and shocks) was not accompanied by significant changes in the extracellular citrulline level in the medial segment of the nucleus accumbens as compared with the baseline level prior to behavioral testing (Fig. 3; $F_{(11,55)} = 0.25$, $p = 0.99$). Intergroup comparisons showed that during this test, the increase in the citrulline level in animals of experimental group 1 (not given blocker) was significantly greater than the change in the citrulline level in rats of experimental group 3 (given 7-nitroindazole) (Fig. 3; $F_{(11,228)} = 3.5$, $p < 0.001$).

Presentation to animals of experimental group 4 ($n = 8$) of the chamber in which they had previously acquired the conditioned reflex fear reaction (combination of tone and shocks), led to an increase in the extracellular citrulline level in the lateral segment of the nucleus accumbens as compared with the individual baseline level (Fig. 4; $F_{(11,77)} = 3.5$, $p < 0.001$). The magnitude of this increase was no different from changes in citrulline levels during this test in the medial segment of the nucleus accumbens in rats of experimental group 1 (Fig. 4; two-factor ANOVA, $F_{(11,252)} = 0.6$, $p = 0.8$).

Presentation to rats of control group 2 ($n = 8$) of the conditioned reflex chamber in which they had previously been exposed to shocks and the tone was accompanied by prolonged (30 min) increases in the extracellular citrulline level in the medial segment of the nucleus accumbens (Fig. 5; $F_{(11,77)} = 7.2$, $p < 0.001$) as compared with baseline) with a peak (156 ± 17%, $t = 8.2$, $p < 0.001$) during the first 5 min in the chamber. Intergroup comparison showed that this increase overall was greater than the increase in the citrulline level in animals of experimental group 1 during presentation of the chamber in which they had been presented with the sound and pain stimuli, but in this case simultaneously (Fig. 5; $F_{(11,252)} = 2.2$, $p = 0.013$). Comparison at specific time points showed that these differences resulted from more significant increases in citrulline levels in the first five minutes in the chamber ($t = 2.6$, $p = 0.016$) and longer-last-

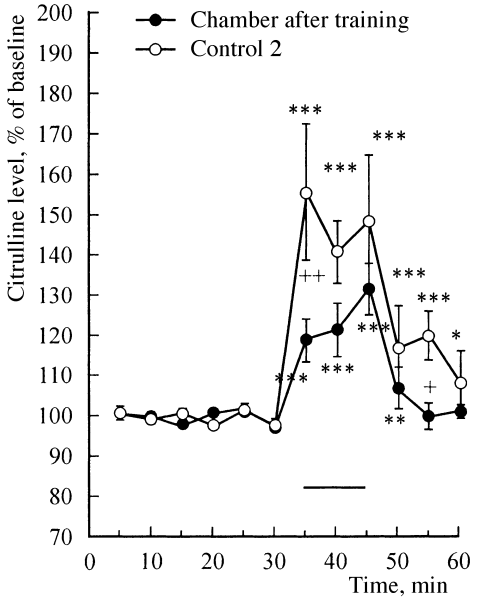

Fig. 5. Changes in extracellular citrulline levels in the medial segment of the nucleus accumbens on presentation of the chamber to animals previously subjected to combined (Chamber after training) or non-simultaneous (Control 2) presentation of the tone and shocks in the chamber. For further details see caption to Fig. 2.

ing changes in citrulline levels in animals of control group 2 as compared with rats of experimental group 1 (t = 3.0, p = 0.006).

DISCUSSION

The unstable chemical compound nitric oxide (NO) is an intercellular messenger in the CNS and at the periphery [7]. NO is synthesized from arginine by an enzymatic reaction catalyzed by NO synthase [8]. The amino acid citrulline is a co-product of this reaction formed in equimolar ratio with NO [8] but has significantly greater chemical stability. This feature of NO metabolism has been used for developing new methods of evaluating its production. In particular, our studies in recent years have shown that extracellular citrulline assessed by in vivo intracerebral microdialysis may reflect the activity of NO synthase in the nucleus accumbens [1, 3, 15], predominantly because of the contribution of the neuronal isoform of this enzyme [3].

Neuronal NO synthase in the nucleus accumbens is present in a small population of interneurons which constitute the basis of the NOergic system of this structure [6]. The activity of these neurons is controlled by hippocampal glutamatergic influences and NMDA glutamate receptors [6, 15]. Given that the integrity of the hippocampal formation is critically important for forming conditioned reflex fear reactions to contextual stimuli [5, 13], the NOergic system of the nucleus accumbens, controlled by hippocampal influences, may also take part in regulating this conditioned reflex behavior. There are no reports to this effect in the literature. The results obtained in the present study support this suggestion, demonstrating that execution of the conditioned reflex fear reaction to contextual stimuli is accompanied by increases in the extracellular citrulline level in the medial segment of the nucleus accumbens and that this increase was completely blocked by local administration of a neuronal NO synthase inhibitor. These data provide evidence that increases in extracellular citrulline levels in the medial segment of the nucleus accumbens on presentation of a potentially dangerous context result from local enhancement of neuronal NO synthase activity, which in all probability leads to increases in NO production in this area of the brain. These changes are evidently not associated with activation induced by transfer of the animal, as they are not seen in control rats when they are placed in the chamber not associated with pain stimulation.

The nucleus accumbens has two segments, a medial "shell" and a lateral "core," which at the functional level have both similarities and differences [9–11]. In particular, data have been obtained showing that both segments of the nucleus accumbens may be involved in organizing responses to contextual danger signals, possibly because of the regulation of the different components of this reaction. Increased dopamine release has been demonstrated in both segments in animals in contexts of danger [10]. The results obtained in the present study supplement arguments supporting this point of view and demonstrating that presentation of contextual shock-associated signals evokes essentially equal increases in the extracellular citrulline level in the medial (mainly the shell) and lateral (mainly the core) segments of the nucleus accumbens.

It should be noted that the extent of activation of the NOergic system in the medial segment of the nucleus accumbens on presentation of contextual danger signals was significantly lower than on combined presentation of sound and contextual danger signals, which is apparent as more significant increases in the extracellular citrulline level during the second test than the first. We suggest that these differences may reflect the additional contribution of conditioned sound signals to activation of the NOergic system of the nucleus accumbens, which is consistent with data on the involvement of the medial segment of the nucleus accumbens in organizing responses to conditioned sound signals previously combined with shocks [9]. A further cause of such significant differences in the extent of NOergic activation may be the fact that during acquisition of the conditioned reflex fear reaction, the animal learned to associate pain stimulation with two conditioned signals simultaneously – the sound and the contextual stimulus. This may weaken the formation of associations (including at the neurochemical level) between the shocks and the chamber in favor of associations between the shocks and the conditioned sound signal. This study yielded data sup-

porting this suggestion. Placing of the animal into the chamber in which they had previously been presented with electric shocks and sound signals non-simultaneously and in random order (making the association between the shock and the sound less likely) was accompanied by more significant and long-lasting increases in extracellular citrulline levels in the medial segment of the nucleus accumbens as compared with values in animals previously exposed to combined presentation of shocks and sounds in the same chamber. These data suggest that in the medial segment of the nucleus accumbens, competition to influence the NOergic system of this structure between the sound and contextual signals occurs during training. Further verification of this suggestion requires additional investigations.

Overall, the new data obtained in these studies provide evidence that being in a dangerous context activates neuronal NO synthase in the medial segment of the nucleus accumbens, leading to increases in the extracellular citrulline level and, probably, to enhanced NO production in this area of the brain.

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REFERENCES

1. S. A. Saveliev, N. S. Renkina, and N. B. Saulskaya, "A sensitive method for assay of citrulline for the in vivo monitoring of nitric oxide production in the CNS," *Ros. Fiziol. Zh. im. I. M. Sechenova*, **91**, No. 5, 587–591 (2005).
2. S. A. Saveliev and N. B. Saulskaya, "Extracellular citrulline levels in the nucleus accumbens during the acquisition and extinction of a classical conditioned reflex to pain reinforcement," *Zh. Vyssh. Nerv. Deyat.*, **56**, No. 1, 86–94 (2006).
3. N. B. Saulskaya, S. A. Saveliev, N. A. Solovieva, and N. V. Fofonova, "NO synthase-dependent increases in extracellular citrulline levels in the nucleus accumbens during an emotional conditioned response," *Ros. Fiziol. Zh. im. I. M. Sechenova*, **92**, No. 6, 700–708 (2006).
4. J. M. Bossert, S. Y. Lie, L. Lu, and Y. Shaham, "A role of ventral tegmental area glutamate in contextual cue-induced relapse to heroin seeking," *J. Neurosci.*, **24**, No. 47, 10726–10730 (2004).
5. M. S. Fanselow, "Contextual fear, gestalt memories, and the hippocampus," *Behav. Brain Res.*, **110**, No. 1, 73–81 (2000).
6. S. J. French, G. P. Ritson, S. Hidaka, and S. Totterdell, "Nucleus accumbens nitric oxide immunoreactive interneurons receive nitric oxide and ventral subicular afferents in rats," *Neuroscience*, **135**, 121–131 (2005).
7. J. Garthwaite, "Concepts of neural nitric oxide-mediated transmission," *Eur. J. Neurosci.*, **27**, 2783–2802 (2008).
8. R. G. Knowles, M. Palacios, R. M. Palmer, and S. Moncada, "Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase," *Proc. Natl. Acad. Sci. USA*, **86**, 5159–5162 (1989).
9. L. Levita, J. W. Dalle, and T. W. Robbins, "Nucleus accumbens dopamine and learned fear revisited: a review and some new findings," *Behav. Brain Res.*, **137**, 115–127 (2002).
10. R. C. R. Martinez, A. R. Oliveira, C. E. Macedo, V. A. Molina, and M. L. Brandao, "Involvement of dopaminergic mechanisms in the nucleus accumbens core and shell subregions in the expression of fear conditioning," *Neurosci. Lett.*, **446**, 112–116 (2008).
11. G. E. Meredith and S. Totterdell, "Microcircuits in the nucleus accumbens' shell and core involvement in cognition and reward," *Psychobiology*, **27**, 165–186 (1999).
12. M. R. Milad, S. P. Orr, P. K. Pitman, and S. L. Rauch, "Context modulation of memory for fear extinction in humans," *Psychophysiology*, **42**, No. 4, 456–464 (2005).
13. R. G. Phillips and J. E. Le Douxe, "Different contribution of amygdale and hippocampus to cued and contextual fear conditioning," *Behav. Neurosci.*, **106**, No. 2, 274–285 (1992).
14. G. Riedel, N. R. Harrington, G. Hall, and E. M. Macphail, "Nucleus accumbens lesions impair context, but not cue, conditioning in rats," *Neuroreport*, **8**, No. 11, 2477–2481 (1997).
15. N. B. Saulskaya and N. V. Fofonova, "Effects of N-Methyl-D-aspartate on extracellular citrulline level in the rat nucleus accumbens," *Neurosci. Lett.*, **407**, No. 1, 91–95 (2006).