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Microdialysis Unveils the Role of the α_2-Adrenergic System in the Basolateral Amygdala during Acquisition of Conditioned Odor Aversion in the Rat

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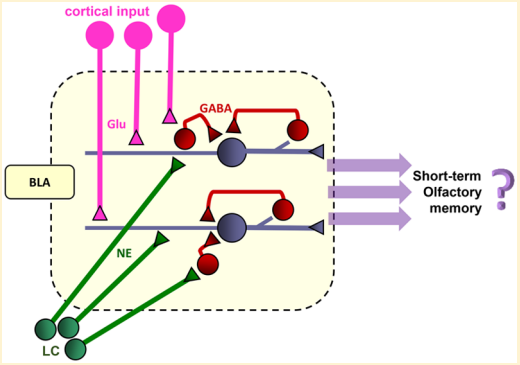
ABSTRACT: Previous work has shown that β-adrenergic and GABAergic systems in the basolateral amygdala (BLA) are involved in the acquisition of conditioned odor aversion (COA) learning. The involvement of α_2-adrenoreceptors, however, is poorly documented. In a first experiment, male Long-Evans rats received infusions of 0.1 μg of the selective α_2-antagonist dexefaroxan (Dex) in the BLA before being exposed to COA learning. In a second experiment, levels of norepinephrine (NE) were analyzed following Dex retrodialysis into the BLA. While microdialysis data showed a significant enhancement of NE release in the BLA with Dex, behavioral results showed that pre-CS infusion of Dex impaired, rather than facilitated, the acquisition of COA. Our results show that the NE system in the BLA is involved in the acquisition of COA, including a strong α_2-receptor modulation until now unsuspected. Supported by the recent literature, the present data suggest moreover that the processes underlying this learning are probably mediated by the balanced effects of NE excitatory/inhibitory signaling in the BLA, in which interneurons are highly involved.



KEYWORDS: *α_2-Adrenoceptors, norepinephrine, conditioned odor aversion, basolateral amygdala, trace conditioning, acquisition, memory, microdialysis*

Conditioned odor aversion (COA) is a robust learned association that involves avoidance of an ingested odorized-tasteless solution (the conditioned stimulus, CS) after its association with toxicosis (the unconditioned stimulus, US). In contrast to the very well-known conditioned taste aversion paradigm, COA can be obtained only if the interval between odor presentation and toxicosis is in the range of a few minutes, suggesting that the memory trace of the odor is subject to rapid decay.[1] Previous studies investigating the neurobiological substrate of COA showed that the basolateral nucleus of the amygdala (BLA) plays a prominent role in the processes underlying the odor-toxicosis association. Our previous studies clearly implicated the BLA GABAergic system in the modulation of the memory trace of the olfactory CS during COA acquisition. Intra-BLA infusion of muscimol (a $GABA_A$ agonist) immediately before or after CS presentation during acquisition prevented taste-potentiated COA,[2] whereas post-CS infusion of bicuculline methiodide (a $GABA_A$ antagonist) enabled COA acquisition with a long CS-US interval.[3] In addition, post-CS infusions of muscimol in the BLA during COA acquisition disrupted facilitation induced by entorhinal cortex (EC) lesion.[4]

Several findings indicate that the role of the BLA during COA could also be modulated by the action of the ascending noradrenergic system. First, many studies reported that post-training intra-amygdala and intra-BLA infusion of noradrenergic agonists or antagonists, respectively, enhances and impairs consolidation of fear-based memory (see ref 5 for review). Furthermore, animals with higher norepinephrine (NE) levels after training display better retention performance.[6] Second, the amygdala receives strong NE input from the locus coeruleus, a structure involved in associative olfactory learning[7,8] by acting directly on both the olfactory bulb[9] and higher-order cortical (prefontal cortex) olfactory processing

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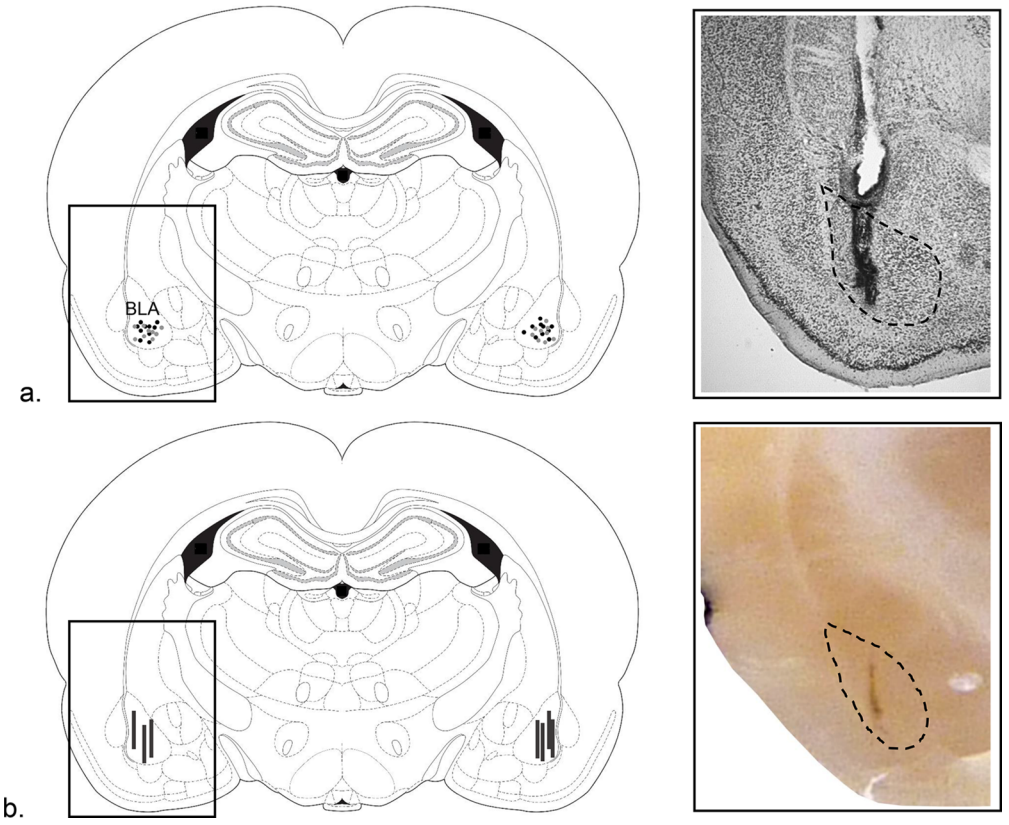

Figure 1. (a) Schematic representation of the amygdaloid complex showing the injection site locations in the Dex (gray circles) and control (black circles) experimental groups. The solid line indicates the position of the representative photomicrograph (right panel) illustrating a typical cannula needle track in the basolateral amygdala (BLA). (b) Schematic representation of the amygdaloid complex showing the probe locations in the group retrodialyzed with Dex. The solid line indicates the position of the representative photomicrograph (right panel) illustrating a typical probe track in the BLA.

systems.[10] Third, control of the olfactory memory trace during COA acquisition was shown to depend on functional interaction between the EC and the BLA,[4] and an in vitro electrophysiological study showed that NE affects the synaptic responses of BLA and EC neurons through distinct and combined action in both structures.[11] Fourthly, behavioral studies showed that infusion of the β-adrenergic antagonist propranolol into the BLA disrupted COA acquisition.[12]

The amygdala contains a high density of β- and α-adrenoceptors.[13,14] Like the β-adrenoceptor, the α_2-adrenoceptor subtype is located pre- and postsynaptically and activated by NE.[15,16] In addition to β-adrenoceptor influences, α-adrenoceptors in the amygdala, and more precisely in the BLA, are involved in the modulation of memory consolidation.[17,18] Taken together with the role of the β-adrenergic system in the BLA during COA acquisition, these data argue for a role of the α_2-adrenergic system in COA learning. To test this hypothesis, the present study aimed at investigating the effects of the pre- and postsynaptic α_2-adrenergic receptor blockade on COA learning. Respective role of each type of receptor in the effects observed with local infusion of the antagonist dexefaroxan hydrochloride (Dex) on COA is then discussed.

RESULTS AND DISCUSSION

Data from four rats in the behavioral study were discarded because of misplacement of the cannula tip location (n = 1), or because dialysate sample levels revealed unstable or below detection limit of NE in the microdialysis study (n = 3). Final group sizes were as follows: Behavioral study: control (n = 10); Dex (n = 9); microdialysis study: Dex (n = 7). Figure 1 illustrates typical cannula tip and probe placements.

Figure 2 shows that conditioned odor aversion (COA) was differentially acquired by the groups as a function of the treatment. As indicated in the figure, the aversion index calculated during test 1 was close to 0.25 for the control group indicating that these animals acquired a strong COA with a 15

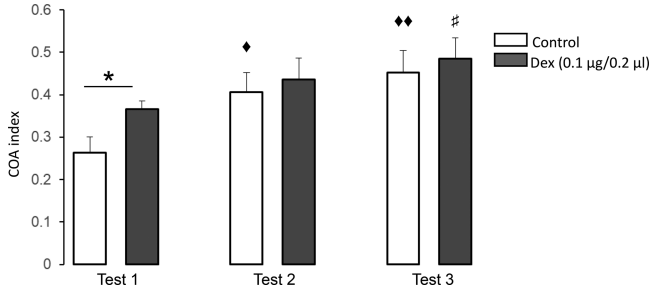

Figure 2. Effects of infusions of vehicle (control, white bars, n = 10) and dexefaroxan (Dex, black bars, n = 9) into the basolateral amygdala 5 min before acquisition of the COA learning. Bars represent COA index (±SEM) calculated as the "mean intake during the test/mean total intake (conditioning + test)". COA = conditioned odor aversion. *p < 0.05 between group comparison during test 1. ♦p < 0.05 and ♦♦p < 0.01 compared with the COA index obtained in test 1. #p < 0.05 compared with the COA index in test 1.

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min CS-toxicosis interval. In addition, the performance calculated during the two successive testing sessions showed a rapid extinction of COA performance with an index reaching 0.45 ± 0.05 in the third test. In contrast, COA index obtained in the Dex-injected group was close to 0.4 during test 1, indicating a relative impairment of COA. Moreover, the successive testing sessions induced an extinction with a COA index reaching 0.49 ± 0.05 in the third testing session. Statistical analysis confirmed this observation with a significant effect of treatment ($F(1,17) = 5.47$, $p < 0.05$). Within-group comparison revealed an effect of extinction session on COA performances in the control group ($F(2,27) = 4.60$, $P < 0.05$) but not in the Dex-injected group ($F(2,24) = 2.23$; $P > 0.05$). Further posthoc comparisons showed significant differences in the COA indexes between test 1 and tests 2 and 3 in the control group ($P < 0.05$ and 0.01 successively), and a difference between the indexes obtained in test 1 and test 3 in the Dex-injected group ($P < 0.05$). The fact that COA extinguished in the Dex group suggests that blockade of α_2-adrenoceptors in the BLA reduced but did not prevent COA learning.

Figure 3 illustrates the values (mean ± SEM) of NE levels measured in 15 min dialysate fractions from BLA collected in

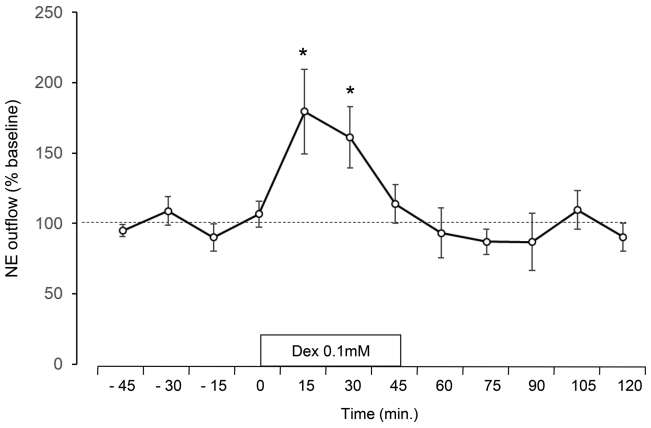

Figure 3. Effect of dexefaroxan (Dex) retrodialysis on norepinephrine (NE) levels in 15 min dialysate samples from the BLA in anesthetized animals ($n = 7$). Solid line with circles indicates mean (±SEM) level of NE in the animals retrodialyzed for 45 min with CSF containing 0.1 mM Dex. NE levels (mean ± SEM) are expressed as a percentage of baseline (levels in 4 samples immediately preceding the start of the retrodialysis ; $T = 0$ min). *$p < 0.05$ compared to baseline and NE levels measured from $T = 45$ to 120 min).

the group retrodialyzed with Dex. As shown in the figure, a significant increase in NE levels occurred within the first 15 min sample following the start of Dex retrodialysis (179.53 ± 30.08%) in the animals. These levels remained significantly elevated in the following sample (161.12 ± 21.88% at $T = 30$ min) before returning to baseline 15 min after the end of infusion. One-way ANOVA with repeated measures confirmed this description and showed a significant effect of treatment ($F(11,88) = 6.18$; $P < 0.001$). Further posthoc t tests revealed that NE levels in the samples collected at $T = 15$ and 30 min were significantly higher when compared to baseline levels (from $T = -45$ to $T = 0$ min; from $P < 0.05$ to $P < 0.01$) and also higher when compared to NE levels measured in the samples collected from $T = 45$ to $T = 120$ (from $P < 0.05$ to $P < 0.01$).

The present results show that retrodialysis of Dex enhanced NE levels in the first 15 min following drug infusion (Figure 3). These results are in accordance with previous pharmacological data reporting that retrodialysis of 0.1 mM of idazoxan (selective α_2-adrenoceptor antagonist) was effective in significantly enhancing NE overflow in the prefrontal cortex.[19,20] Moreover, in reference to the effect of Dex retrodialysis in vivo,[21] the present data suggest that microinjection of Dex in the BLA before our acquisition procedure strongly increased NE release.

Beta- and α-adrenoceptor subtypes are expressed at high levels in the amygdala (see ref 22 for review). NE release in the BLA has complex effects including direct alteration of pyramidal projection neurons (PN) excitability and presynaptic modulation of GABA release from interneurons.[23−26] First, noradrenergic signaling modulates excitatory transmission in the BLA via activation of postsynaptic β- and α_1-adrenoceptors onto excitatory PN[27] and via α_2-adrenoceptors, which inhibit PN excitability.[11,28] Second, local inhibitory feed-back projecting interneurons make perisomatic synapses on PN.[29] These feed-back projecting interneurons express α_1-adrenoreceptors, the activation of which increases GABA release onto PN.[23] In addition, another population of interneurons expressing β-receptors is located at the external capsule border (lateral paracapsular cells). These interneurons make synapse on PN distal dendrites and are activated by cortical afferents, thus providing feed-forward inhibition.[26,30,31] Taken these data together, it appears that BLA activity is modulated through complex balanced excitatory and inhibitory effects of NE and impaired COA performance observed in the present study was probably due to a shift in the effect of NE on this balance in the BLA network activity. In a situation in which an increased NE release elicited by Dex injection shifted the balance toward enhancement of BLA excitability during the acquisition of the task, the postsynaptic effect of NE on β- and α_1-receptors on the glutamatergic transmission of PN (and BLA output) should have enhanced COA performance. Accordingly, several studies showed that (i) large quantities of NE released in the amygdala, and particularly in the BLA, directly influence learning performance during inhibitory avoidance learning;[6,21] (ii) increased BLA PN excitability is associated with improved acquisition and expression of fear learning;[32−35] and (iii) disinhibition of BLA activity induces long-term potentiation which is associated with fear memory formation.[36−41] However, the fact that we did not observe such an effect on COA learning suggests that the effect of Dex microinjection more probably affected COA through a shift of the balance toward GABAergic-mediated inhibition of BLA excitability. In support of this hypothesis recent studies reported that feed-forward GABergic inhibition onto excitatory PN in the BLA is involved in the modulation of CS pathway enhancement in fear conditioning.[42] In addition, stress-enhanced endogenous NE level, or NE injection in the olfactory bulb, impaired odor recognition memory[43] and locus coeruleus stimulation paired with odor presentation strongly reduced mitral cell responses to odor recognition memory of this odor through a GABAergic pathway.[44] Taken together with recent results showing that hippocampal LTP is influenced by α_2-adrenoceptor activation in the BLA,[45] the present data suggest that the impairing effect of Dex on COA resulted from NE-mediated enhancement of presynaptic GABA release during acquisition, resulting in inhibition of PN firing in the BLA and consequently in downstream structures. Although speculative, this assumption

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Table 1. Representation of the Sequence of Phases Taking Place during the Behavioral Procedure

days 1−3	days 4−9	day 10	day 11	days 12−14
handling	habituation to the drinking schedule (twice a day)	microinfusion and COA conditioning	water	COA test

would have important implications for understanding the neural mechanisms by which the BLA contributes to modulation of olfactory memory.

Extinction test results (Figure 2) showed that COA was reduced but not completely blocked by the infusion of Dex. In the light of the time course of NE release in the BLA observed in the microdialysis experiment, it is suggested that Dex may have affected the (i) sensory processing and/or (ii) the memory formation of the odor during COA acquisition. Concerning the first point, one study of ours showed that intra-BLA infusion of the $GABA_A$ agonist muscimol immediately before the retrieval test (i.e., before CS presentation) did not prevent the expression of COA, thus suggesting that the activation of the GABAergic system in the structure is not involved in sensory processing of the odor CS.[46] Concerning the second point, a broad range of data suggest the impairing effect of Dex may result from a deficit in a memory process underlying the CS-US association. Notably, we have shown that the glutamatergic and GABAergic systems in the BLA are selectively involved in the association between the CS memory trace and the delayed US during COA acquisition.[47] Considering the main facilitation effect of NE on the GABAergic inhibitory response recorded in the BLA PN,[23,25] it is suggested that Dex microinjection affected the memory process underlying the association between the olfactory trace and the delayed US, an effect that was probably mediated, at least in part, by NE action on the GABAergic system in the BLA. In conclusion, the present study strongly suggests that physiologically relevant changes in NE influence odor memory through global inhibitory mode in the BLA network activity.

METHODS

A total of 30 male adult Long-Evans rats (280−300 g at the time of surgery) were used. After arrival, they were housed two per cage in transparent Plexiglas cages (43 × 22 × 16 cm^3) in a temperature-controlled (21 °C) colony room and maintained on a standard 12 h light/dark cycle (lights from 7:00 AM to 7:00 PM) with access to food and water ad libitum. Rats were allowed to become accustomed to the laboratory vivarium for 1 week before surgery. All experimental sessions were carried out during the light portion of the cycle between 11:00 AM and 1:00 PM. All procedures involving animals and their care were strictly under both ongoing national and european community laws and policies. All surgical procedures were conducted under optimal aseptic, analgesic and ethical animal care conditions (see ref 48).

For the behavioral study, 20 rats were anaesthetized by intraperitoneal (i.p.) injection of a mix of ketamine (100 mg/kg)/xylasine (10 mg/kg) and fixed in a stereotaxic frame in a flat skull position. Stainless steel guide cannulae (12 mm long; 23 gauge) were placed bilaterally according to the Paxinos and Watson[49] stereotaxic coordinates: 2.8 mm posterior to bregma, 4.95 mm lateral to the midline, and 6.5 mm ventral to the skull surface. Surgical screws placed in the skull above the frontal and posterior cortices served as anchors. The cannulae were affixed to the skull with dental cement. Stylets were inserted into the guide cannulae and remained there at all times except during injections. All subjects recovered for seven to 11 days after surgery with ad libitum access to food and water. Before the start of the behavioral procedure, rats were handled (3 min/day) and weighed for 3 days (Days 1−3). On the third day, the water bottles were removed in the evening and a 23 h 35 min water deprivation schedule was initiated. The rats had access to water twice a day, once for 15 min (between 11:00 AM and 1:00 PM) and again for 10 min in their home cages (6:00 PM). This drinking schedule lasted throughout the behavioral experiment. Animals were weighed daily to verify their adaptation to the deprivation schedule and the volume of water intake was measured by weighing the bottles before and after each morning drinking sessions. The rats were acclimated to this regimen for 6 days (from day 4 to day 9) before conditioning was started. On the conditioning phase (day 10), animals had access to the olfactory CS (0.01% amyl acetate in tap water in their water bottles) for 15 min. Then the bottles were removed and each animal was injected 15 min later with 0.15 M lithium chloride (10 mL/kg; i.p.). Five minutes before the CS presentation (Pre-CS), rats were microinjected bilaterally as follows: while gently handled, injection needles (30 gauge) were inserted to a depth of 1.5 mm beyond the tips of the guide cannula and connected via polyethylene tubing to two 10-μL Hamilton syringes driven by an automated syringe pump (Carnegie Medicine, Stockholm, Sweden). Injections of 0.2 μL of sterile artificial cerebrospinal fluid (aCSF, Harvard Apparatus) or dexefaroxan hydrochloride (Dex, α_2-adrenoceptor antagonist) dissolved in sterile aCSF (0.1 μg/0.2 μL per side; Sigma) were delivered concurrently in both hemispheres in animals of the Control (n = 10) and Dex (n = 10) groups respectively over a period of 30 s. This concentration was selected on the basis of previous pharmacological data obtained with acute microinfusion of the selective α_2-adrenoceptor antagonist idazoxan in the BLA of rats during inhibitory avoidance learning in the rat.[50] Injection needles were then left in place at each injection site for an additional 30 s period. On day 11, rats had twice access to water bottles in their home cage (15 min between 11:00 AM and 1:00 PM and 10 min at 6:00 PM). On days 12−14, conditioned odor aversion was assessed by presenting the olfactory CS. The successive phases corresponding to the behavioral procedure are summarized in Table 1.

For the microdialysis study, a group of naïve rats (n = 10) was anesthetized by intraperitoneal (IP) injection of urethane (1.62g/kg, i.p.) and fixed in a stereotaxic frame (David Kopf, Tujunga, CA) in a flat skull position. The body temperature was maintained close to 37 °C using a heated underblanket (Harvard Instruments, Les Ulis, France). After being flushed with water, the probes were continuously infused with aCSF throughout the surgical procedure at 1 μL/min. Microdialysis probes were placed randomly in the left or right BLA at the following coordinates according to the atlas:[49] 2.8 mm posterior to bregma, 4.95 mm lateral to the midline, and 8 mm ventral from the dura. A postimplantation delay of 3-h was respected before collection of basal samples. Subsequently, dialysates were collected continuously in the anesthetized animal at a 15 min sampling rate. The dead volume of the tubing was estimated to represent only 6% of the final sampling time, so that the delay between neurochemical data and the behavioral events was considered short enough to not require correction. Each sample was stored and kept at −80 °C before high-performance liquid chromatography (HPLC) analyses. Dexefaroxan hydrochloride was solubilized in aCSF in order to obtain the final 0.1 mM concentration. This concentration was selected on the basis of previous microdialysis data obtained with the use of the selective α_2-adrenoceptor antagonist idazoxan in the medial prefrontal cortex and BLA of rats.[20,21] Animals were retrodialyzed at T0 min for 45 min with Dex (0.1 mM; n = 7). NE concentrations in dialysates were expressed as a percentage of basal levels measured in the four samples immediately preceding the drug infusion procedures ("baseline").

All statistical analyses were done with the Systat 12.0 program. The "aversion index" corresponding to the "mean odorized water intake during the test/mean total intake (conditioning + test)" was analyzed with a one-way ANOVA followed by posthoc t tests. Differences in the percentage baseline values of NE between each sample were analyzed using one-way repeated-measures ANOVA followed by posthoc t tests. For all the statistical comparisons, the significance

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level was set at 0.05. The number of animals per group is indicated in Figures 2 and 3.

After completion of behavioral testing, rats were given an overdose of sodium pentobarbital (100 mg/kg) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The brains were then extracted, postfixed for 4 h in the same fixative (4 °C) and transferred into a 0.1 M phosphate-buffered 30% sucrose solution for about 36−40 h (4 °C). Coronal sections, 20 μm, were cut on a freezing microtome (−21 °C). Brain sections of animals in the behavioral groups were collected and dried at room temperature before being stained with cresyl violet and microscopic determination of injection site; probe location of animals in the microdialysis study was determined directly on frozen sections.

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Author Contributions

L.E.: contributed to the experimental design, behavioral experiments, and manuscript writing. J.-C.C.: contributed to microdialysis experiment and manuscript writing. S.P.: participated to the experimental design and manuscript writing. P.D.-V.: participated to experimental design and manuscript writing. B.F.: designed all experiments and manuscript, supervised research of L.E..

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Notes

The authors declare no competing financial interest.

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