Allosteric modulation of GABA_A receptors in rat basolateral amygdala blocks stress-enhanced reacquisition of nicotine self-administration

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Abstract

Stress is a major determinant of relapse to smoked tobacco. In a rat model, repeated stress during abstinence from nicotine self-administration (SA) results in enhanced reacquisition of nicotine SA, which is dependent on the basolateral amygdala (BLA). We postulate that repeated stress during abstinence causes hyperexcitability of BLA principal output neurons (PN) due to disinhibition of PN from reduced inhibitory regulation by local GABAergic interneurons. To determine if enhanced GABAergic regulation of BLA PNs can lessen the effects of stress on nicotine intake, positive allosteric modulators (PAMs) of GABA_A receptors were infused into the BLA immediately prior to reacquisition of nicotine SA. Three selective PAMs (e.g., NS 16085, DCUK-OEt, DS2) with varied GABA_A subunit specificities abolished the stress-induced amplification of nicotine taking during reacquisition. These studies indicate that highly selective PAMS targeting $\alpha 3$ or δ subunit-containing GABA_A in BLA may be effective in ameliorating the stress-induced relapse to smoked tobacco during abstinence from cigarettes.

Introduction

Nicotine is the principal psychoactive agent in smoked tobacco. My laboratory developed the operant behavioral paradigm for virtually unlimited (i.e., 23 hour) access to nicotine self-administration (SA) in order to model human smoking (1). More recently, we modeled the effects of stress during abstinence from nicotine SA, which is among the most prevalent causes of relapse to smoking. We have shown that repeated stress during abstinence from nicotine SA results in enhanced nicotine SA and enhanced motivation to obtain nicotine following abstinence (2). As such, this is a model for stress-induced relapse to smoked tobacco in humans. Additional experiments showed that circuitry in the basolateral amygdala (BLA) and its connections to nucleus accumbens core are necessary for the expression of stress-enhanced nicotine SA (3).

We postulate that repeated stress during abstinence causes hyperexcitability of BLA principal output neurons (PN) due to disinhibition of PN from reduced inhibitory regulation by local GABAergic interneurons (4, 5). These interneurons activate GABA_A receptors on BLA principal neurons (PN) that project to multiple brain regions, including monosynaptic glutamatergic efferents to median spiny neurons in the nucleus accumbens.

GABA_A receptors are ligand-gated pentameric chloride channels principally assembled from α , β , and γ or δ subunits (6). BLA PNs express a variety of GABA_A receptors (GABA_A) containing $\alpha 2$, $\alpha 3$, β , $\gamma 2$ and δ subunits (7-9).

Based on the foregoing hypothesis, we determined if enhanced GABAergic regulation of BLA PNs can lessen the effects of stress on nicotine intake. Positive allosteric modulators (PAMs) of GABA_A were infused into the BLA immediately prior to the reacquisition of nicotine SA by rats that had previously been stressed during abstinence from nicotine. PAMs targeting different GABA_A subtypes present in BLA were evaluated: NS16085 (4-chloro-3-{6-[5-(2-hydroxypropan-2-yl)-1H-1,3-benzodiazol-1-yl]pyridin-2-yl}benzonitrile) is highly specific for the benzodiazepine binding site on $\alpha 2/\alpha 3$ subunit-containing GABA_A (10); DCUK-OEt (5,7-dichloro-4-([diphenyl carbamoyl] amino) quinoline-2-ethyl carboxylate) binds to a novel, benzodiazepine and flumazenil insensitive GABA_A site that requires specific subunit combinations (i.e., $\alpha 1$ or $\alpha 5$, $\beta 2$ or $\beta 3$, $\gamma 2$ or δ) (11); and DS2 (4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl benzamide) binds exclusively to δ subunit-containing GABA_A (12, 13).

We found that all three GABA_A PAMs diminished the stress-induced amplification of nicotine intake following abstinence from the drug. This suggests that more than one subtype of GABA_A modulates BLA neuronal activity, which is affected by chronic stress during abstinence from nicotine. Highly selective PAMs, such as DCUK-OEt, may be beneficial in reducing stress-related relapse to cigarette smoking.

Results

To verify the location of microinfusions into BLA, random animals that had previously been implanted with guide cannulae were injected with AAV-CAMKII-mCherry; brains were obtained after completion of behavioral studies. Figure 1 demonstrates the diffusion and expression throughout BLA of a representative infusion of AAV-CAMKII-mCherry. Fluorescence is largely confined to and detectable throughout most of BLA. Figure 2 shows BLA schematics of injection sites in rats infused with each PAM.

Figure 3 shows the effects of bilateral BLA infusions of NS16085 (20 μ M) on the reacquisition (ReAq) of operant nicotine SA after chronic exposure to stress during abstinence. Reacquisition of nicotine SA was assessed during three consecutive daily sessions, beginning 24h after the 4th stress.

NS16085 (20 μ M per side) or vehicle was infused 30 min before nicotine SA sessions on d1 and d2 of reacquisition (arrows). In the group receiving vehicle, active lever presses and nicotine infusions increased after repeated stress on day 1 of reacquisition compared to acquisition (Acq) ($F=10.3$, $p=0.006$ and $F=3.5$, $p=0.080$ for active lever and infusion, respectively). The interaction of stage [reacquisition vs acquisition] x treatment was significant for both lever and infusion ($F=11.8$, $p=0.004$ and $F=10.1$, $p=0.006$ for lever and infusion, respectively). In the vehicle group, active lever presses and infusions were both significantly greater on reacquisition day 1 compared to acquisition ($p < 0.00006$; Tukey). Additionally, on reacquisition day 1, treatment with NS 16085 blocked the enhancing effects of repeated stress on both lever presses and infusions observed in the vehicle group ($p < 0.003$; Tukey).

Figure 4 shows the effects of infusing DCUK-OEt (5 or 20 μ M) or vehicle into BLA bilaterally 30 min before nicotine SA sessions on d1 and d2 of reacquisition (ReAq, arrows). Chronic stress increased active lever presses and infusions in the groups receiving vehicle or DCUK-OEt 5 μ M during reacquisition, but not in the DCUK-OEt 20 μ M group (2-way ANOVA: $F=13.7$, $p=0.004$ and $F=14.1$, $p=0.002$ for lever and infusion, respectively). A

significant interaction of stage x treatment was also found for active presses and infusions ($F=15.2$, $p=0.003$ and $F=18.3$, $p=0.0009$ for lever and. Infusions, respectively). Lastly, on d 1 of reacquisition, DCUK-OEt (20 μ M) significantly reduced both lever presses and infusions compared to vehicle (#, $p < 0.001$; Tukey), without affecting the underlying operant nicotine SA. Thus, enhanced activation of BLA GABA_A induced by the novel PAM, DCUK-OEt, blocked the stress-enhanced component of nicotine SA.

In Figure 5, DS2 (20 μ M), a δ subunit-specific PAM, or vehicle was infused into BLA bilaterally 30 min before nicotine SA sessions on d1 and d2 of reacquisition. Chronic stress increased active lever presses and infusions in the vehicle group during reacquisition, but not after DS2 (2-way ANOVA: $F=8.1$, $p=0.013$ and $F=5.7$, $p=0.032$ for lever and infusion, respectively; *, $p<0.000001$ for lever and infusion in vehicle reacquisition vs acquisition, Tukey). A significant interaction of stage x treatment was also found for active presses and infusions ($F=9.3$, $p=0.009$ and $F=25.5$, $p=0.0002$ for lever and. Infusions, respectively). On days 1 and 2 of reacquisition, DS2 significantly reduced both lever presses and infusions compared to vehicle (#, $p=0.009$ for lever and $p=0.0002$ for infusion; Tukey), without affecting the underlying operant nicotine SA. Thus, enhanced activation of BLA GABA_A

induced by the highly selective PAM, DS2, was sufficient to prevent the stress-enhanced component of nicotine SA.

Since GABA_A containing $\alpha 3$ subunits (7) may mediate the foregoing behavioral effects of NS 16085 on BLA function, we sought to determine if these subunits are also targeted by DCUK-OEt. Electrophysiology studies have identified several α subunits that interact with this compound, although $\alpha 3$ was not evaluated (11). Therefore, the effects of DCUK-OEt on GABA_A assembled from $\alpha 3$ in combination with $\beta 2\gamma 2$ or $\beta 3\gamma 2$ were evaluated.

The results in Figure 6 show the potentiation by DCUK-OEt of inhibitory currents induced by GABA (EC₁₀) in $\alpha 3$ subunit-containing recombinant rat GABA_A isoforms expressed by *Xenopus laevis* oocytes. As previously reported with $\alpha 1\beta 3\gamma 2$ (11), DCUK-OEt effectively and potently potentiated the inhibitory currents generated by $\alpha 3\beta 2\gamma 2$ (n=4) and $\alpha 3\beta 3\gamma 2$ (n=5): EC₅₀=0.38 and 0.13 μ M, respectively. This potentiation was not blocked by Flumazenil (1 μ M or 10 μ M), an antagonist specific for the GABA_A benzodiazepine site. Therefore, the potentiation of inhibitory currents by DCUK-OEt depends on interactions with a novel site on $\alpha 3$ subunit-containing GABA_A.

Discussion

These experiments demonstrate that BLA GABA_A modulation by PAMs can abolish the stress-enhanced reacquisition of nicotine SA. Positive allosteric modulators, augmenting GABA_A activation by GABA, completely inhibited the stress-enhanced intake of nicotine and the increase in active lever presses during reacquisition of nicotine taking without altering the underlying level of either parameter achieved during the final 3d of acquisition. These observations lend support to the hypothesis that stress-enhanced reacquisition of nicotine SA involves diminished GABAergic regulation of local BLA circuits.

The following observations show that local BLA GABAergic neurons are affected by chronic stress that, in turn, alters the GABAergic regulation of BLA principal glutamatergic output neurons. These PNs, which innervate NAcc monosynaptically, are required for expression of stress-enhanced reacquisition of nicotine SA (3). 1. Dopaminergic efferents from VTA modulate a parvalbumin-positive subset of GABA interneurons within BLA (14,15), in turn, inhibit the activity of BLA PNs (7,16). 2. Extra-synaptic $\alpha 3$ -containing GABA_A mediate tonic inhibition of BLA PNs; excitability of these neurons was markedly reduced by TP003, a GABA_A PAM that binds at the

benzodiazepine site (7). 3. Stresses, such as chronic restraint, increase BLA Da release, which inhibits parvalbumin-positive GABA interneurons (17,18). Stress also sensitizes amygdaloid Da efflux to drug conditioned stimuli (19).

Taken together, the foregoing observations support the hypothesis that stress-induced increase in BLA Da decreases GABA release from parvalbumin positive BLA interneurons, thereby disinhibiting BLA PNs and promoting release of glutamate from BLA efferents to multiple brain regions. The current experimental observations are consistent with this, showing that bilateral BLA delivery of NS 16085, a highly specific PAM of $\alpha 2/\alpha 3$ subunit-containing GABA_A, and DCUK-OEt, a novel PAM that interacts with several GABA_A including those assembled from $\alpha 2$ subunits (11) and $\alpha 3$ subunits, as shown in Fig. 6, blocked expression of the stress-enhanced component of nicotine SA during reacquisition.

BLA is regulated by two types of GABAergic currents (for review: 20). In the mouse, fast, phasic postsynaptic currents primarily occur through GABA_A, containing $\alpha 2$ and some $\alpha 1$ subunits, but not $\alpha 3$ subunits. In contrast, tonic extra-synaptic inhibition, detected in BLA GABA interneurons and most principal neurons, depends largely on $\alpha 3$ subunit-containing GABA_A

receptors in PNs and on non- $\alpha 3$ variants in interneurons (7). These tonic currents are generated by atypical non-desensitizing GABA_A receptors with high affinity for low levels of extra-synaptic GABA that are sensitive to benzodiazepines and TP003 (7). Blocking tonic currents increases neuronal excitability by shifting the resting membrane potential to more positive values. Conversely, amplifying the currents with TP003 produces a more negative resting potential. Thus, low concentrations of extra-synaptic GABA maintain a persistent background inhibitory conductance that affects the cellular and network E/I balance, thereby regulating intrinsic excitability and the integration of synaptic inputs (21).

There are two non-exclusive mechanisms that are likely to explain the effects of NS 16085 on stress-enhanced reacquisition of nicotine SA. NS 16085 may enhance the tonic inhibitory regulation of BLA PCs by increasing the activation of atypical extra-synaptic $\alpha 3$ -containing GABA_A, thereby reducing the excitability of PCs (8). Alternatively, NS 16085 may amplify the postsynaptic inhibitory currents on PCs by increasing the activity of $\alpha 2$ -containing GABA_A - the primary subunit mediating these postsynaptic currents. In light of the fact that TP003, another $\alpha 2/\alpha 3$ subunit-specific PAM,

inhibits excitability of BLA PC neurons from wild type but not $\alpha 3$ -knockout mice, the second mechanism appears unlikely (8).

DCUK-OEt, which modulates the activity of $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ - and $\alpha 5$ -containing GABA_A would most likely interact with GABA_A that are similar to those affected by NS 16085, i.e., those containing extra-synaptic $\alpha 3$ subunits. In addition to modulating $\alpha 3$ -containing GABA_A, DCUK-OEt also might interact with $\alpha 1$ -containing GABA_A, although these are a minor component of the GABA_A controlling post-synaptic BLA PC currents; moreover, $\alpha 1$ -containing GABA_A do not interact with NS 16085.

DS2 effectively blocked the stress enhanced component of nicotine SA during reacquisition. This finding implicates an additional set of GABA_A that are assembled from δ instead of γ subunits and are devoid of benzodiazepine binding sites. Therefore, extra-synaptic δ -containing GABA_A are insensitive to NS 16085. GABA_A that contain δ subunits mediate tonic, extra-synaptic inhibitory currents, and are usually assembled from $\alpha 4$ and $\alpha 6$ subunits (13). Modulation of these GABA_A by DCUK-OEt appears unlikely, since this PAM

does not bind to $\alpha 4\delta$, although its interaction with $\alpha 6$ -containing GABA_A is unknown (11).

In summary, three GABA_A PAMs were shown to block the stress-enhanced reacquisition of nicotine SA that depends on BLA (2). Based on the GABA_A subunit specificity of NS 16085 and DCUK-OEt, and the inhibition of BLA PN excitability by $\alpha 3$ -containing GABA_A, we propose that tonic, extra-synaptic $\alpha 3$ -containing GABA_A are the primary BLA targets of both PAMs. The potency and efficacy of DCUK-OEt shown in our electrophysiological analyses of $\alpha 3$ -containing GABA_A are consistent with this idea. The efficacy of DS2 in our behavioral model also indicates that a second subset of δ subunit-containing GABA_A modulate BLA excitability. These studies indicate that highly selective GABA_A PAMs may be effective in ameliorating the stress-induced relapse to smoked tobacco during abstinence from cigarettes.

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Figures

Figure 1. Photomicrographs of AAV-CAMKII-mCherry expression in BLA: (L) BLA (DIC; AP -2.8, ML $\pm$ 4.6, DV -7.4) shows small amount of old hemorrhage (yellow) surrounding tip of implanted guide cannula just above BLA; tip of injection cannula is shown by arrow; (R) mCherry fluorescence expressed in BLA neurons.

Figure 2. BLA schematics of injection sites in rats infused with NS 16085 (L), DCUK-OEt (middle) or DS2 (R).

Figure 3. Effect of bilateral BLA infusion of NS16085 on the reacquisition (ReAq) of nicotine SA after chronic exposure to stress during abstinence. Reacquisition of nicotine SA was assessed 24h after 4th stress. NS16085 (20 μ M) was infused into BLA 30m before nicotine SA sessions on d1 and d2 of ReAq. Active Lever and Nicotine Infusion: Stage [Acquisition (Acq) v ReAq] and Stage x Treatment (repeated measures ANOVA): $F=10.3$, $p=0.006$ and $F=11.8$, $p=0.004$, respectively for lever; $F=3.5$, $p=0.080$ and $F=10.1$, $p=0.006$, respectively for infusion. * $p < 0.00006$ v Acq within group, # $p < 0.003$ NS v vehicle at d1 (Tukey).

Figure 4. Effect of bilateral BLA infusion of DCUK-OEt on the reacquisition (ReAq) of operant nicotine SA after chronic exposure to stress during abstinence. Reacquisition of nicotine SA (FR5) was assessed 24h after 4th stress. DCUK (5 and 20 μ M; 300nl/side in 5% DMSO) was infused into BLA 30m before nicotine SA sessions on d1 and d2. Active Lever and Nicotine Infusion: Stage [Acquisition (Acq) v ReAq] and Stage x Treatment (repeated measures ANOVA): $F=13.7$, $p=0.004$ and $F=15.2$, $p=0.003$, respectively for lever; $F=14.1$, $p=0.002$ and $F=18.3$, $p=0.0009$, respectively for infusion.

Figure 5. Effect of bilateral BLA infusion of DS2 on the reacquisition (ReAq) of operant nicotine SA after chronic stress during abstinence. Reacquisition of nicotine SA (FR5) was assessed 24h after 4th stress. DS2 (20 μ M; 300nl/side) was infused into BLA 30m before nicotine SA sessions on d1 and d2. Active Lever and Nicotine Infusion: Stage [Acq] v ReAq] and Stage x Treatment (repeated measures ANOVA): $F=8.1$, $p=0.013$ and $F=9.3$, $p=0.009$ for lever; $F=5.7$, $p=0.032$ and $F=25.5$, $p=0.0002$ for infusion, respectively. *, $p<0.000001$ for lever and infusion in vehicle reacquisition vs acquisition (Tukey). Reacquisition Vehicle vs DS2: #, $p=0.009$ for lever and $p=0.0002$ for infusion (Tukey).

Figure 6. Effect of DCUK-OEt on the potentiation of $\alpha 3$ subunit-containing GABA_A inhibitory currents induced by GABA (EC₁₀) in recombinant rat GABA_A expressed by *Xenopus laevis* oocytes. Top (L) panel shows DCUK-OEt potentiation of inhibitory currents in $\alpha 3\beta 2\gamma 2$ (n=4) and $\alpha 3\beta 3\gamma 2$ (n=5): EC₅₀=0.38 and 0.13 μ M, respectively. Top (R) panel shows that potentiation by DCUK-OEt 1 μ M was not blocked by Flumazenil 1 μ M or 10 μ M. Bottom panels show the inhibitory currents induced by GABA (EC₁₀; Clamp = -70mV) and potentiated by increasing concentrations of DCUK-OEt, and the failure of Flumazenil to block this potentiation.

Materials and Methods

Animals and surgeries

Adult male Sprague-Dawley rats (250 g, Harlan, Madison, WI) were housed in a reversed 12:12 h light/dark cycle and provided with standard rat chow and water *ad libitum*. Rats were anesthetized with ketamine/xylazine (94.3/0.11 mg/kg, respectively, i.p.) and guide cannulas (26 gauge, Plastic one, Roanoke, VA) were stereotaxically implanted bilaterally into basolateral amygdala (BLA) approximately 3 weeks prior to use. For BLA, coordinates from bregma (flat skull) are: anteroposterior (AP) = -2.56 mm, mediolateral (ML) = ± 4.6 , dorsoventral (DV) = -7.4 mm (22). After 2 d of recovery, jugular catheters were implanted. All procedures conformed to NIH guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center.

Nicotine self-administration

Rats were given extended access to nicotine SA (23 h/d, 7 d/week) without food deprivation or prior training. The nicotine SA procedure was conducted as previously described (1). Briefly, after 3 d of recovery post jugular surgery, rats were placed into an operant conditioning chamber located inside a sound-attenuating environmental chamber. The operant

conditioning chamber contained two horizontal levers, and a green cue light above each lever was illuminated when nicotine was available during SA sessions. Lever presses were recorded and syringe pumps were controlled by computers and interfaces, using L2T2 software (Coulbourn Instruments, Allentown, PA, USA). Nicotine SA started immediately after initiation of the dark cycle. Pressing the active lever, randomly designated, elicited an intra-jugular injection of nicotine solution (0.03 mg/kg, in 50 μ l/0.81 s, free base, pH 7.2-7.4). To avoid over-dosing from nicotine, each injection initiated a 7s timeout during which the green cue light above the lever was extinguished and nicotine was unavailable. Pressing the alternate (inactive) lever had no programmed consequence. The final hour of the 12 h lights-on cycle (i.e., 9:00–10:00 AM) was reserved for animal husbandry, refreshing the nicotine solution, and data recording. The patency of jugular catheters was checked periodically and assessed by injection of methohexital sodium (10 mg/ml, 0.1 ml; Par Pharmaceuticals, Spring Valley, N.Y.) Rats with occluded catheters were excluded from the experiment.

Experimental design of behavioral studies

In all experiments, the protocols for acquisition of nicotine SA, abstinence, restraint stress and reacquisition of nicotine SA were conducted as

previously described (2). Briefly, rats acquired nicotine SA at escalating fixed ratio (FR) schedules of reinforcement: an increasing number of active lever presses was required to receive a single injection of nicotine. Rats initiated nicotine SA on an FR 1 schedule: 1 active lever press yields 1 nicotine injection. This schedule continued for 7 d, increasing to FR 2 (for 3 d) and then FR 5 (for 7 d). After acquisition, animals were withdrawn from nicotine in their home cages (not operant conditioning chambers) for 8 d (without nicotine, levers, and cue lights). This interval was selected since the majority of attempts to quit smoking fail within the first 8 days (23) and most withdrawal symptoms, in humans and rats, peak and subside within the first week (24,25). Restraint stress was administered in a 6.5 cm diameter plastic cylinder for 30 min between 9:00–10:00 AM on d 1, 3, 5, and 7 of abstinence. 24h after the final stress, animals were returned to operant conditioning chambers for the reacquisition of nicotine SA under an FR5 schedule for a total of 3 d.

Prior to (i.e., 30 min) designated reacquisition sessions, the following compounds were infused into BLA (5 and 20 μ M in 0.3 μ l per side over 6 min), using a microsyringe infusion pump connected to an injection cannula (33 gauge) extending 1.5 mm beyond the tip of the guide cannula: NS16085 (4-

chloro-3-{6-[5-(2-hydroxypropan-2-yl)-1H-1,3-benzodiazol-1-yl]pyridin-2-yl}benzonitrile; 10), a highly $\alpha 2/\alpha 3$ subunit-specific positive allosteric modulator of GABA_A receptors; DCUK-OEt (5,7-dichloro-4-([diphenyl carbamoyl] amino) quinoline-2-ethyl carboxylate; 11), binds a novel, benzodiazepine and flumazenil insensitive GABA_A site requiring specific GABA_A subunit combinations (i.e., $\alpha 1$ or $\alpha 5$, $\beta 2$ or $\beta 3$, $\gamma 2$ or δ); DS2 (4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl benzamide; 12,13), binds exclusively to δ subunit-containing GABA_A; or vehicle. Injection cannulas remained in place 5 min to ensure diffusion of drug.

Electrophysiological studies of recombinant rat GABA_A

Recombinant rat GABA_A $\alpha 3\beta 2\gamma 2$ and $\alpha 3\beta 3\gamma 2$ were expressed in *Xenopus laevis* oocytes, as previously described (26). Electrophysiological studies were performed according to the methods of Borghese et al. (11). Unless otherwise stated, the washout period between the application of compounds was 3 minutes. Compounds were applied sequentially: GABA (1mM) followed by 15 min washout; GABA (EC₁₀) tested twice (clamp= -70mV); DCUK-OEt at increasing concentrations (0.01-1.0 μ M) + GABA (EC₁₀); Flumazenil (1.0 or 10 μ M) + GABA (EC₁₀) + DCUK-OEt (1 μ M) followed by

15 min washout; Zn test to validate the incorporation of γ subunits into GABA_A. Multiple compounds were all co-applied.

Histology

After the final d of reacquisition, rats were anesthetized with ketamine/xylazine (94.3/0.11 mg/kg, respectively, i.p.) perfused with transcardiac paraformaldehyde, and then brains were immediately fixed in paraformaldehyde (4% in 0.1 M phosphate buffer) for 24h, dehydrated in 20% sucrose followed by 30% sucrose for 2 x 24 h, sectioned at 40 μ m thickness (CM1850, Leica, Nussloch, Germany), and mounted on pre-cleaned slides. The location of each microinjection cannula was determined by light microscopy.

Data analysis and statistics

Both active lever presses and nicotine injections, during the final 3 d of acquisition of nicotine SA and during the 2 d of reacquisition of nicotine SA (FR5 schedule), were expressed as a percentage of the baseline, defined as the average number of presses or injections during the final 3 d of acquisition. The behavioral data obtained during reacquisition were analyzed by repeated measures 2-way ANOVA, using SPSS Statistics 22 (IBM,

Armonk, New York), with day of reacquisition designated as a within-subjects factor and drug treatment designated as a between-subjects factor. One-way ANOVA and multiple *posthoc* comparisons (Tukey) were used to compare various time intervals within the same treatment group. Data were expressed as mean $\pm$ SEM. Statistical significance was assigned at $p < 0.05$.

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