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Repeated pretreatment with amphetamine sensitizes increases in cortical acetylcholine release

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Abstract *Rationale:* Previous studies on the attentional effects of repeated psychostimulant administration in rats suggested the possibility that these effects are mediated via increases in the efficacy of psychostimulants to stimulate cortical acetylcholine (ACh) release. Furthermore, neurochemical data have raised the possibility that increases in nucleus accumbens (NAC) dopamine (DA) release trans-synaptically increase the excitability of basal forebrain corticopetal cholinergic projections, thereby supporting speculations about relationships between the effects of repeated psychostimulant administration on NAC DA and cortical ACh release. *Objectives:* To determine whether repeated exposure to amphetamine would potentiate the stimulating effects of the drug on cortical ACh and NAC DA efflux. *Methods:* Rats were implanted with microdialysis guide cannula in the medial prefrontal cortex and the shell region of the ipsilateral NAC. Amphetamine (2.0 mg/kg i.p.) or saline (0.9%) was administered every other day for 10 days, for a total of five injections. ACh and DA efflux and locomotor activity were measured on the day of the first and last injections of this pretreatment regimen. All animals were retested following a challenge dose of amphetamine (2.0 mg/kg i.p.) given 10 and 19 days after the last pretreatment injection. *Results:* The initial injections of amphetamine stimulated ACh and DA efflux and locomotor behavior in both groups. The pretreatment with amphetamine potentiated the ability of the drug to stimulate cortical ACh efflux on day 19 of the withdrawal period. The pretreatment with amphetamine also increased the effects of the challenge dose on motoric activity on day 10. Pretreatment with amphetamine did not result in a significant augmentation of the amphetamine-induced increase in DA efflux in the NAC. *Conclusions:* Pretreatment with

amphetamine sensitizes the ability of amphetamine to stimulate cortical ACh efflux. These results support the hypothesis that sensitized release of cortical ACh mediated the previously observed hyperattentional impairments in amphetamine pretreated rats. Sensitized cortical ACh release following repeated exposure to psychostimulants may mediate the overprocessing of addictive drug-related stimuli, thus contributing to repeated compulsive addictive drug use.

Key words Cortex · Acetylcholine · Nucleus accumbens · Dopamine · Amphetamine · Microdialysis · Addiction

Introduction

Repeated administration of psychostimulants produces potentiated or sensitized neurobehavioral effects relative to those seen following a single administration of the drug. At the neuronal level, repeated exposure to amphetamine or cocaine results in sensitized release of dopamine (DA) in the nucleus accumbens (NAC; Kalivas and Stewart 1991; Paulson and Robinson 1995; Robinson et al. 1988; Vezina 1993; Wolf et al. 1993). At the behavioral level, sensitization to stimulants has traditionally been defined as enhanced motoric activity (Kalivas and Stewart 1991; Robinson and Becker 1986). Several authors have suggested that the neuronal plasticity mediating these sensitized effects, particularly at the level of dopaminergic transmission within the NAC, contribute to the drug craving that is crucial to repeated addictive drug use, and this has promoted an interest in psychostimulant sensitization as an animal model for studying drug abuse (Berridge and Robinson 1998; Robinson and Becker 1986; Robinson and Berridge 1993; Sarter and Bruno 1999).

While the neurochemical consequences of repeated psychostimulant administration have received considerable attention over the past decade, considerably less attention has been devoted to revealing the altered cognitive processes that may contribute to compulsive drug

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use. One such component is the biased overprocessing of previously neutral or irrelevant stimuli that have become associated with drug use (Robinson and Berridge 1993; Sarter and Bruno 1999). Such dysfunctions in attentional processing may mediate the subjective experience of drug craving and thus contribute to compulsive addictive drug use. There is initial support for such attentional effects of repeated psychostimulant administration. For example, Deller and Sarter (1998) demonstrated that repeated administration of systemic amphetamine in animals performing a sustained attention task resulted in amphetamine-sensitized increases in the number of false alarms, that is 'claims' for signals in non-signal trials. This effect was interpreted as reflecting the animals' adoption of a riskier criterion in reporting signals or, in more general terms, the overprocessing of the stimulus situation (see also Crider et al. 1982). Increased false alarm rates have also been suggested to result from addictive drug use in humans (Damos and Parker 1994).

There is considerable evidence supporting a role for the basal forebrain and cortical cholinergic transmission in the mediation of attentional processing. Cortical cholinergic afferents arising from the basal forebrain have been proposed to "act as a gating mechanism channeling behaviorally relevant sensory information toward components of the limbic system" (Mesulam 1990). Thus, the basal forebrain cholinergic system may modulate the processing efficacy of sensory information and mediate attentional processes (Everitt and Robbins 1997; Sarter and Bruno 1997). Cortical cholinergic transmission has been shown to be necessary for performance in a sustained attention task (McGaughy and Sarter 1998; McGaughy et al. 1996). Moreover, drugs that reduce acetylcholine (ACh) release by positively modulating the GABAergic inhibition of basal forebrain neurons (Moore et al. 1995a,b) impair performance in the sustained attention task (Holley et al. 1995). Conversely, drug-induced negative modulation of the GABAergic inhibition of these neurons, produced by infusions of benzodiazepine receptor inverse agonists into the basal forebrain, were found to increase the number of false alarms in attentional task-performing rats (Holley et al. 1995). Finally, the magnitude of cortical ACh release is related to the extent of the attentional demands in this vigilance task (Himmelheber et al. 2000). Based on these data, the ability of repeated administration of amphetamine to increase the false alarm rate in rats performing a sustained attention task has been speculated to reflect the drug's well-established capacity to increase cortical ACh release (Day and Fibiger 1992; Day et al. 1994; Arnold et al. 2000). Furthermore, the effects of amphetamine on cortical ACh release have been hypothesized to be related to the amphetamine-induced increases in DA receptor activity within the NAC (Day et al. 1994; but see Arnold et al. 2000). Consequently, this experiment tested the hypothesis that the repeated exposure to amphetamine results in the potentiated or sensitized stimulation of cortical ACh release. The present experiment employed an amphetamine pretreatment

schedule similar to that used in the experiment by Deller and Sarter (1998). The effects of amphetamine on cortical ACh and NAC DA efflux, and on locomotor activity, were assessed during the pretreatment period as well as after 10 and 19 days of withdrawal after completion of the pretreatment.

Materials and methods

Subjects

Adult male Fischer-344/Brown Norway F1 hybrid rats (Harlan Sprague-Dawley, Indianapolis, Ind., USA), weighing between 250 and 350 g, were housed in a temperature-controlled (23°C) colony room with a 12:12 h light:dark cycle (lights on at 06:30 a.m.). Prior to stereotaxic surgery, animals were housed in pairs in stainless steel hanging cages. Food and water were available ad libitum. On the night prior to surgery, animals were individually housed in standard plastic cages (24×45×20 cm high) with pine shavings, where they were housed for the duration of the experiment. All housing, surgery, experimentation, and euthanasia were performed in accordance with Ohio State University Animal Care and Use Committee-approved protocols and the *U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals*.

Stereotaxic surgery

Animals were handled and habituated to the microdialysis bowls (35×38 cm diameter) and test rooms for 4 days prior to surgery. On the morning of surgery, animals were anesthetized with ketamine (100.0 mg/kg i.p.) and xylazine (6.0 mg/kg i.p.) prior to surgery and then placed in a stereotaxic apparatus. Stainless steel microdialysis guide cannula (0.5 mm O.D.; Bioanalytical Systems, W. Lafayette, Ind., USA) were implanted into the medial prefrontal cortex (mPFC; prelimbic and infralimbic cortex) and the shell region of the ipsilateral NAC. Hemispheres were counterbalanced. For the mPFC, the arm of the apparatus was angled 10° anterior to vertical at the following coordinates: 3.0 mm anterior, 0.8 mm lateral, 1.1 mm below dura. For the NAC shell, the arm was positioned 15° posterior to vertical at 0.3 mm posterior, 1.1 mm lateral, 5.7 mm below dura (all coordinates were relative to Bregma; Paxinos and Watson 1986). Cannulae were fixed using skull screws and dental cement. Following surgery, animals were returned to their home cages and allowed to recover for 3 days prior to the first dialysis session.

Experimental design and drug administration protocol

Following surgery, animals were divided into two groups. Beginning on the 4th day following surgery, one group of animals ($n=8$) received one injection of *d*-amphetamine sulfate (2.0 mg/kg i.p., dissolved in 0.9% saline) every other day for a total of five drug injections, and saline (0.9%) on "off" days. The other group ($n=7$) received daily saline throughout the pretreatment period. The habituation period and saline or amphetamine injections during the pretreatment period were conducted in the test room while animals were contained within the microdialysis test bowls (35×38 cm diameter). On microdialysis days, one amphetamine- and one vehicle-preexposed animal were tested simultaneously. Animals were dialyzed on the day of the first administration of amphetamine/saline and the final day of amphetamine/saline administration during the pretreatment period. Following the pretreatment period, animals were placed in the test bowls daily and received one daily saline injection. All animals received "challenge" injections of amphetamine (2.0 mg/kg i.p.) on day 10 and day 19 of the withdrawal period. Microdialysis sessions were conducted on both challenge days.

Microdialysis procedure

Microdialysis was conducted using a repeated perfusion paradigm with each animal tested on four sessions. On microdialysis days, animals were placed in the testing chamber for 30 min prior to probe insertion. Concentric microdialysis probes (2.0 mm membrane tips; Bioanalytical Systems) were inserted into the mPFC and NAC guide cannula and continuously perfused with artificial cerebrospinal fluid (aCSF), containing (in mM): NaCl 166.5, NaHCO₃ 27.5, KCl 2.4, CaCl₂ 1.2, Na₂SO₄ 0.5, KH₂PO₄ 0.5, glucose 1.0, pH=6.9. The aCSF in the mPFC also contained a low concentration (0.05 μM) of the acetylcholinesterase inhibitor neostigmine bromide. Probes were attached to a dual channel liquid swivel (Instech, Plymouth Meeting, Pa., USA) and perfused at a rate of 1.25 μl/min for 3 h prior to baseline collections, resulting in stable ACh efflux (Moore et al. 1992) and DA efflux (Nakamura et al. 1992; Westerink et al. 1987) that is highly dependent on neuronal impulses. Collections were taken every 15 min, and each baseline period consisted of four collections. Following the baseline period, amphetamine (2.0 mg/kg i.p.) or saline was administered and collections were taken for an additional 2 h. The collection vials from the NAC contained 5.0 μl of an antioxidant solution (0.05 N PCA, 200 μM sodium bisulfite, and 1.0 mM EDTA) to minimize degradation of DA during the collection intervals. Collections were stored at -80°C prior to neurochemical analysis.

Measurement of motor activity

Motor activity was measured using an 11-point scale modified from Ellinwood and Balster (1974). Ratings were based on the following scale: (1) lying down, eyes closed, (2) lying down, eyes open, (3) standing or hunching in place, (4) in-place activities (for example, grooming, sniffing), (5) locomotion about bowl, (6) above average levels of movement/activity, (7) hyperactivity (running/jumping), (8) repetitive exploration with normal activity, (9) repetitive exploration with hyperactivity, (10) restricted/stereotypy, (11) dyskinesia. Behavior was rated for 30 s at the middle and conclusion of each collection interval, and an average score was assigned to that interval.

Neurochemical analyses

ACh levels in the mPFC dialysate were determined using high-performance liquid chromatography with electrochemical detection (HPLC-ED). A volume of 12.0 μl from each collection was injected (Model 540 autosampler; ESA, Chelmsford, Mass, USA) and ACh and choline were separated (0.5 ml/min) by a C-18 carbon polymer column (ESA) using a sodium phosphate mobile phase [in mM: Na₂HPO₄ 100.0, TMAcI 0.5, 1-OSA 2.0; 0.005% of the antimicrobial reagent MB, (ESA) pH=8.0]. ACh and choline were then hydrolyzed on a post-column enzyme reactor and converted to hydrogen peroxide (Potter et al. 1983). This was detected using a "peroxidase-wired" glassy carbon electrode. The detection limit for ACh under these conditions was approximately 10.0 fmol/12.0 μl injection.

DA in NAC dialysates was determined using HPLC-ED (ESA). A volume of 20.0 μl from each collection was injected (Model 540 autosampler) and DA was separated (0.6 ml/min) with a carbon polymer column (ESA) using a sodium phosphate mobile phase (75.0 mM NaH₂PO₄·H₂O, 2.0 mM 1-OSA, 25 μM EDTA, 200 μl TEA, 18.0% methanol, pH=5.6). DA was detected using a coulometric electrode (ESA). The detection limit under these conditions was approximately 3.0 fmol/20.0 μl injection.

Verification of probe placements

At the conclusion of the experiment, animals received a sublethal dose of sodium pentobarbital and were transcardially perfused with 0.2% heparin and 10.0% formalin. Brains were stored in

10.0% formalin at 4°C for 24 h and transferred to a 30.0% sucrose phosphate buffer solution until sectioning. Coronal sections (40 μm) were mounted on gelatin-coated slides and stained with cresyl violet. Probe placements were verified using the atlas of Paxinos and Watson (1986), and any animals determined to have probe placements outside the mPFC or shell of the ipsilateral NAC were removed from the study.

Statistical analyses

Statistical analyses were conducted separately for the two treatment groups (i.e., vehicle- and amphetamine-pretreated). Separate, yet parallel, analyses were conducted on the ACh and DA efflux data. Given the repeated perfusion paradigm, the initial analysis evaluated whether *basal* ACh or DA efflux differed across the four microdialysis sessions. Thus, a mixed factor two-way analysis of variance (ANOVA) with GROUP (vehicle pretreated, amphetamine pretreated) as a between-subject factor and SESSION (first through fourth) as a within-subject factor was conducted on the mean of the median baseline values for ACh (pmol/12 μl) and DA (fmol/20 μl) efflux.

Data from each group were subsequently expressed as a percent change from the median baseline and evaluated using an overall two-way ANOVA consisting of within-subject variables of SESSION (first vehicle or amphetamine, second vehicle or amphetamine, first amphetamine challenge, second amphetamine challenge) and TIME (last baseline through 120 min postinjection). Significant overall effects of SESSION were followed by a series of two-way ANOVAs in which the effects of a specific session were compared with those of another session. In some cases, one-way ANOVAs, within a particular session, were conducted with TIME as a repeated measure in order to analyze initial changes in ACh or DA efflux. *Post hoc* analyses were conducted using *t*-tests for dependent means. Data from the locomotor ratings were analyzed using the non-parametric Wilcoxon sign rank test (Keppel 1991). The nature of this test restricts the comparison to a single repeated measure. Thus, comparisons between different dialysis sessions were conducted on either median baseline activity or at 45 min postinjection (preliminary observations revealed this to be the timepoint of maximal activity). Significance for all tests was defined as $P < 0.05$.

Results

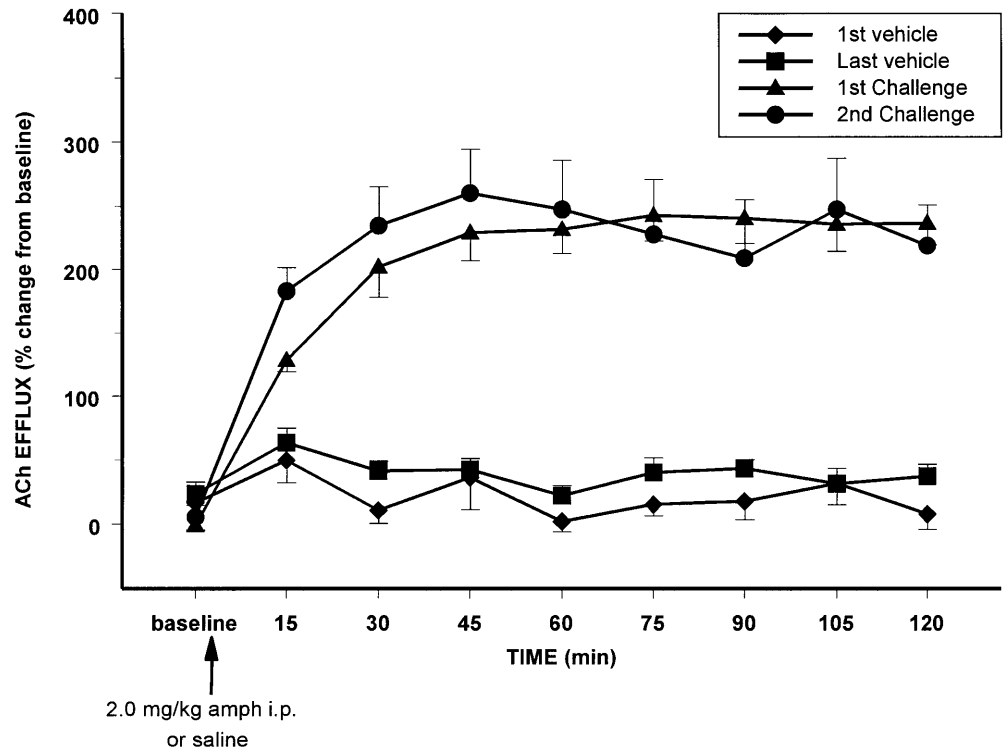
Probe placements

Histological analyses confirmed that the tips of the two microdialysis probes were located within the mPFC and shell region of the ipsilateral NAC in all subjects reported below.

Basal cortical ACh efflux

Overall, basal ACh efflux did not differ between controls (0.063±0.007 pmol/12 μl; mean ± SEM) and rats pretreated with amphetamine (0.057±0.005; $F_{1,39}=0.445$, $P=0.516$). There was a significant effect of repeated microdialysis sessions on basal ACh efflux ($F_{3,39}=14.513$, $P < 0.001$). *Post hoc* analyses revealed that this effect of session was a result of ACh efflux in session 1 (0.092±0.01) being greater than in session 2 (0.052±0.005; $t_{14}=4.504$, $P < 0.001$), session 3 (0.056±0.006; $t_{14}=3.330$, $P=0.005$), and session 4 (0.038±0.007; $t_{14}=4.191$, $P=0.001$). Importantly, there was no significant interac-

Fig. 1 Cortical acetylcholine (ACh) efflux (mean \pm SEM), expressed as a percent change from baseline, in control rats pretreated with vehicle (0.9% saline i.p.) and then subsequently challenged with amphetamine (2.0 mg/kg i.p.). Rats ($n=7$) were dialyzed on the first and last days of the pretreatment regimen and then 10 and 19 days later. Administration of the saline vehicle had no effect on cortical ACh efflux. As expected, amphetamine markedly stimulated cortical ACh efflux. This stimulation was comparable between the first and second drug injections. Mean (\pm SEM) basal ACh efflux (pmol/12 μ l), for sessions 1–4, were 0.108 ± 0.016 , 0.052 ± 0.007 , 0.057 ± 0.008 , and 0.035 ± 0.007 , respectively



tion between GROUP and SESSION ($F_{3,39}=1.887$, $P=0.148$), indicating that this effect of session was identical in animals pretreated with amphetamine or saline. Consequently, all subsequent analyses on cortical ACh efflux were conducted on values expressed as a percent change from the median session baseline.

Amphetamine-stimulated cortical ACh efflux in rats pretreated with vehicle

Figure 1 summarizes cortical ACh efflux in vehicle-pretreated rats as a function of dialysis session. An overall ANOVA revealed that ACh efflux differed markedly across the four dialysis sessions as evidenced by a significant effect of SESSION ($F_{3,18}=37.727$, $P<0.001$) and a SESSION \times TIME interaction ($F_{21,144}=11.958$, $P<0.001$). An analysis of the first pretreatment session revealed that the vehicle injection did not affect cortical ACh efflux (TIME, $F_{8,48}=1.167$, $P=0.338$). There was no significant difference in ACh efflux between the first and last vehicle injection sessions ($F_{1,6}=3.431$, $P=0.113$).

The initial 'challenge' with amphetamine on day 10 of the withdrawal period (withdrawal from saline) produced a marked and prolonged increase in cortical ACh efflux (TIME, $F_{8,48}=63.436$, $P<0.001$). ACh efflux differed between the first challenge and last vehicle session (SESSION, $F_{1,6}=78.729$, $P<0.001$; SESSION \times TIME interaction, $F_{8,48}=44.258$, $P<0.001$). Similar differences were seen between the second challenge with amphetamine and the last vehicle session (SESSION, $F_{1,6}=28.400$, $P=0.002$; SESSION \times TIME, $F_{8,48}=12.260$,

$P<0.001$). There was, however, no difference in the magnitude nor duration of stimulated cortical ACh efflux between the first and second amphetamine 'challenges' (SESSION, $F_{1,6}=0.203$, $P=0.668$).

Stimulated cortical ACh efflux in rats pretreated with amphetamine

Figure 2 illustrates the effects of amphetamine on cortical ACh efflux in rats that were pretreated with amphetamine. An overall ANOVA revealed that cortical ACh efflux varied significantly over the four dialysis sessions (SESSIONS, $F_{3,21}=9.101$, $P<0.001$). As expected, the initial administration of amphetamine during the pretreatment period resulted in a marked and prolonged increase in ACh efflux as evidenced by a significant effect of TIME ($F_{8,56}=15.841$, $P<0.001$). ACh efflux during the last pretreatment with amphetamine was slightly, but significantly, greater than that seen following the initial administration (SESSION, $F_{1,7}=6.522$, $P=0.038$). The initial withdrawal period of 10 days (i.e., first challenge) did not further increase the effects of amphetamine on cortical ACh efflux beyond that seen during the last pretreatment session ($F_{1,7}=0.018$, $P=0.896$). However, amphetamine administration on day 19 of the withdrawal period (i.e., the second challenge) resulted in a potentiated increase in cortical ACh efflux relative to the last pretreatment (SESSION, $F_{1,7}=13.654$, $P=0.008$) and, importantly, relative to the first amphetamine challenge (SESSION, $F_{1,7}=6.519$, $P=0.038$).

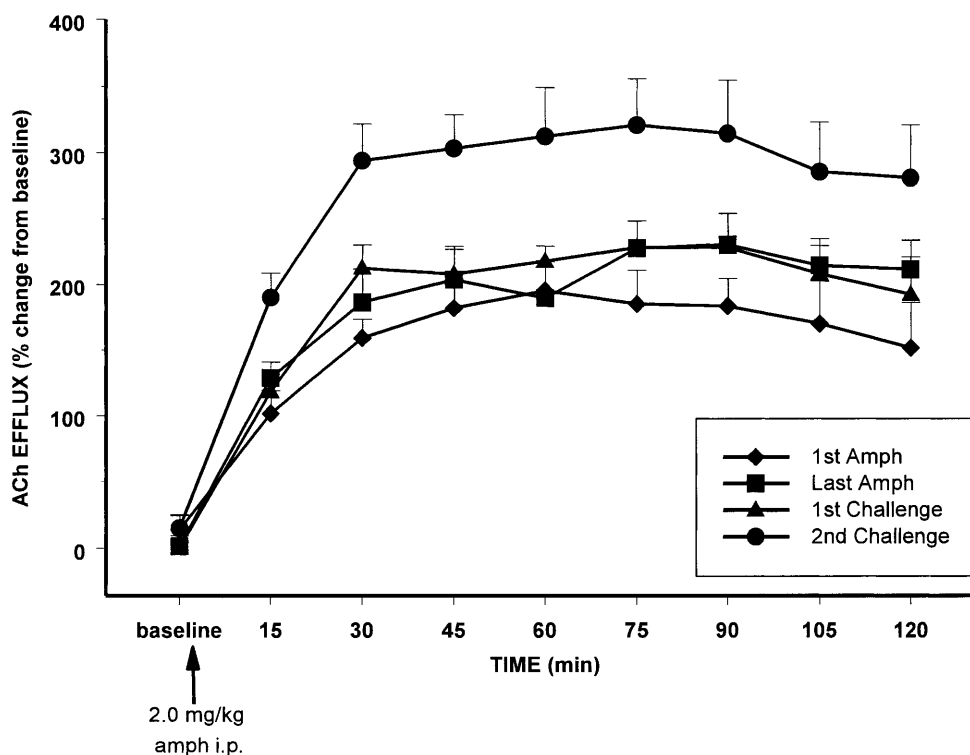


Fig. 2 Cortical ACh efflux (mean \pm SEM), expressed as a percent change from baseline, in rats pretreated with amphetamine (2.0 mg/kg i.p.) and then subsequently challenged with amphetamine (2.0 mg/kg i.p.). Rats ($n=8$) were dialyzed on the first and last days of the pretreatment regimen and then 10 and 19 days into the withdrawal period. The initial administration of amphetamine, as expected, resulted in a marked and prolonged increase in cortical ACh efflux. The final administration of amphetamine during the pretreatment regimen resulted in a modest but significant increase in ACh efflux relative to the initial injection. While the injection of amphetamine during the first withdrawal period did not further increase cortical ACh efflux, efflux during the second withdrawal period was higher than during any previous injection. Mean (\pm SEM) basal ACh efflux (pmol/12 μ l), for sessions 1–4, were 0.077 ± 0.009 , 0.052 ± 0.008 , 0.056 ± 0.008 , and 0.041 ± 0.012 , respectively

Basal NAC DA efflux

Overall, basal DA efflux in NAC did not differ between vehicle- (7.66 ± 0.69 fmol/20 μ l; mean \pm SEM) and amphetamine-pretreated rats (7.73 ± 0.63 ; $F_{1,39}=0.003$, $P=0.956$). There was also no significant effect of repeated microdialysis sessions on basal DA efflux ($F_{3,39}=1.029$, $P=0.390$): session 1 (7.95 ± 0.84), session 2 (8.63 ± 1.04), session 3 (7.46 ± 0.90), and session 4 (6.75 ± 0.91). Likewise, the effects of GROUP and SESSION did not interact ($F_{3,39}=1.996$, $P=0.130$). Consequently, all subsequent analyses on NAC DA efflux were conducted on values expressed as a percent change from the median session baseline.

Amphetamine-stimulated NAC DA efflux in rats pretreated with vehicle

Figure 3 summarizes NAC DA efflux in vehicle-preexposed rats as a function of dialysis session. An overall

ANOVA revealed that DA efflux differed markedly across the four dialysis sessions (SESSION, $F_{3,18}=8.101$, $P=0.001$; SESSION \times TIME, $F_{24,144}=3.881$, $P<0.001$). A one-way ANOVA revealed that injections of saline during the first vehicle session did not affect NAC DA efflux ($F_{8,48}=0.725$, $P=0.669$). A comparison of the first and last vehicle injection sessions revealed similar NAC DA efflux ($F_{1,6}=0.003$, $P=0.958$). The first administration of amphetamine on withdrawal day 10 markedly increased NAC DA efflux (TIME, $F_{8,48}=10.017$, $P<0.001$). A two-way ANOVA revealed that amphetamine's stimulation of NAC DA efflux did not differ between the first (day 10) and second (day 19) challenge (SESSION, $F_{1,6}=1.388$, $P=0.283$; SESSION \times TIME, $F_{8,48}=0.560$, $P=0.805$).

Stimulated NAC DA efflux in rats pretreated with amphetamine

Figure 4 summarizes amphetamine-induced increases in NAC DA efflux in rats that were pretreated with the drug. Administration of amphetamine during the 1st day of the pretreatment regimen markedly increased DA efflux ($F_{8,56}=18.267$, $P<0.001$). While Fig. 4 suggests that there might be a trend for DA efflux to be higher during the challenge sessions than the pretreatment sessions, an overall ANOVA revealed that there were no systematic differences in stimulated DA efflux among the four dialysis sessions ($F_{3,21}=1.004$, $P=0.410$).

Fig. 3 Dopamine (DA) efflux (mean \pm SEM) in the shell region of the nucleus accumbens (NAC), expressed as a percent change from baseline, in control rats pretreated with vehicle (0.9% saline i.p.) and then subsequently challenged with amphetamine (2.0 mg/kg i.p.). Rats ($n=7$) were dialyzed on the first and last days of the pretreatment regimen and then 10 and 19 days later. Administration of the saline vehicle had no effect on NAC DA efflux. As expected, amphetamine markedly stimulated DA efflux. This stimulation was not significantly different between the first and second drug injections. Mean (\pm SEM) basal DA efflux (fmol/20 μ l), for sessions 1–4, were 9.163 \pm 1.723, 8.022 \pm 1.032, 8.165 \pm 1.631, and 5.292 \pm 0.651, respectively

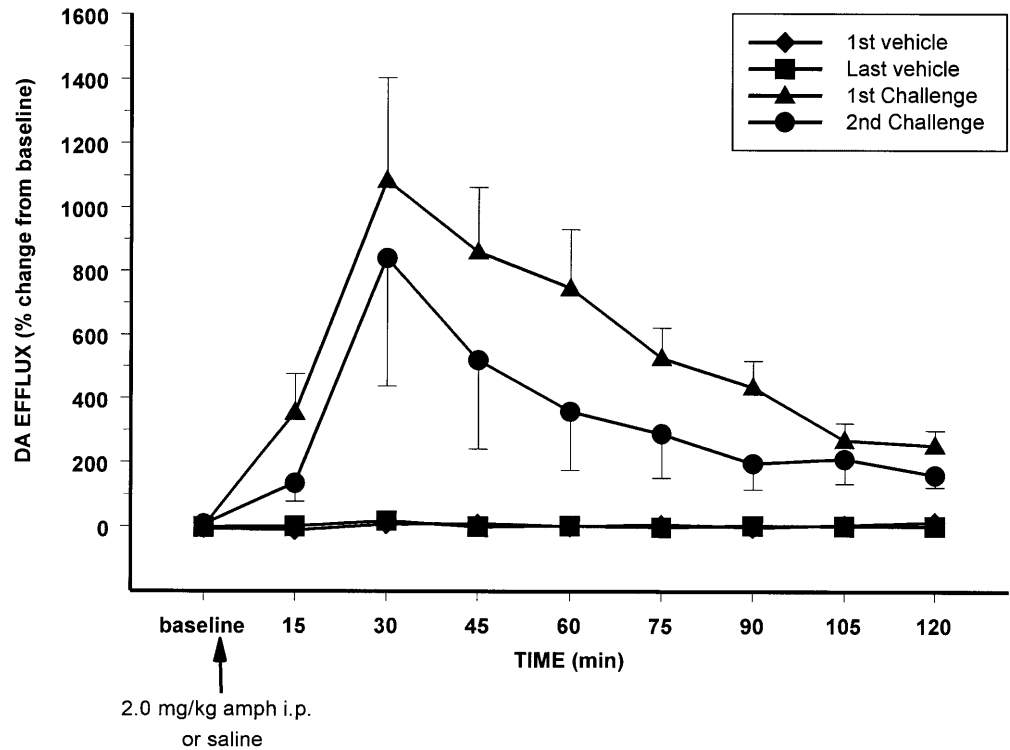
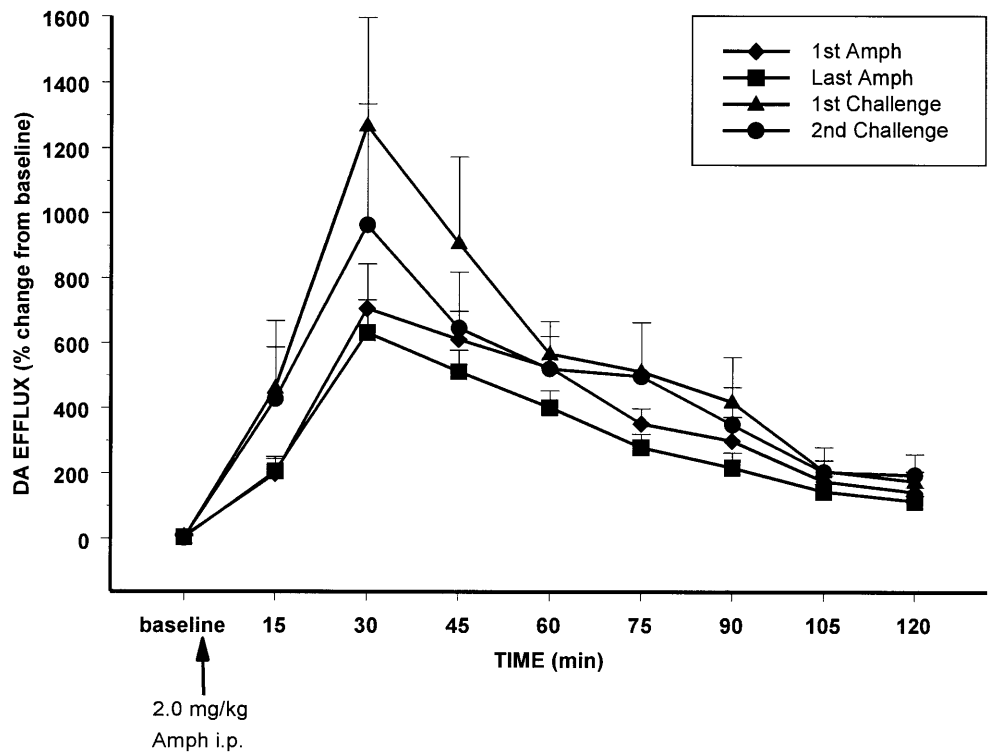


Fig. 4 Accumbens DA efflux (mean \pm SEM), expressed as a percent change from baseline, in rats pretreated with amphetamine (2.0 mg/kg i.p.) and then subsequently challenged with amphetamine (2.0 mg/kg i.p.). Rats ($n=8$) were dialyzed on the first and last days of the pretreatment regimen and then 10 and 19 days into the withdrawal period. The initial and final administration of amphetamine during the pretreatment period, as expected, resulted in a marked and prolonged increase in NAC DA efflux. Despite a trend toward enhanced DA efflux following amphetamine during the withdrawal periods, these differences were not significantly different from the pretreatment sessions. Mean (\pm SEM) basal DA efflux (fmol/20 μ l), for sessions 1–4, were 6.884 \pm 0.316, 9.165 \pm 1.780, 6.840 \pm 0.978, and 8.026 \pm 1.511, respectively



Locomotor activity in rats pretreated with vehicle

Figure 5 illustrates the locomotor activity in rats pretreated with vehicle for the four microdialysis sessions. There were no differences among the four dialysis sessions in baseline activity levels (all P values >0.1). Prior to the injections, animals were awake but typically lying still in

the test bowls. There was no increase in motor activity (baseline vs 45 min) following the saline injections in the first ($Z=-1.089$, $P=0.276$) or last ($Z=0.000$, $P=1.000$) vehicle sessions. Administration of amphetamine resulted in enhanced in-place activity and locomotion in the test bowls. This increased activity at 45 min, relative to baseline, was significant in both the first ($Z=-2.384$, $P=0.017$)

Fig. 5 Mean locomotor ratings in control rats ($n=7$) pretreated with vehicle (0.9% saline i.p.) and then subsequently challenged with amphetamine (2.0 mg/kg i.p.). Injections of saline did not affect motoric behavior, relative to baseline levels, during the two pretreatment sessions. As expected, amphetamine stimulated motoric activity on days 10 and 19, however, there were no differences in the extent of this activation between challenge sessions

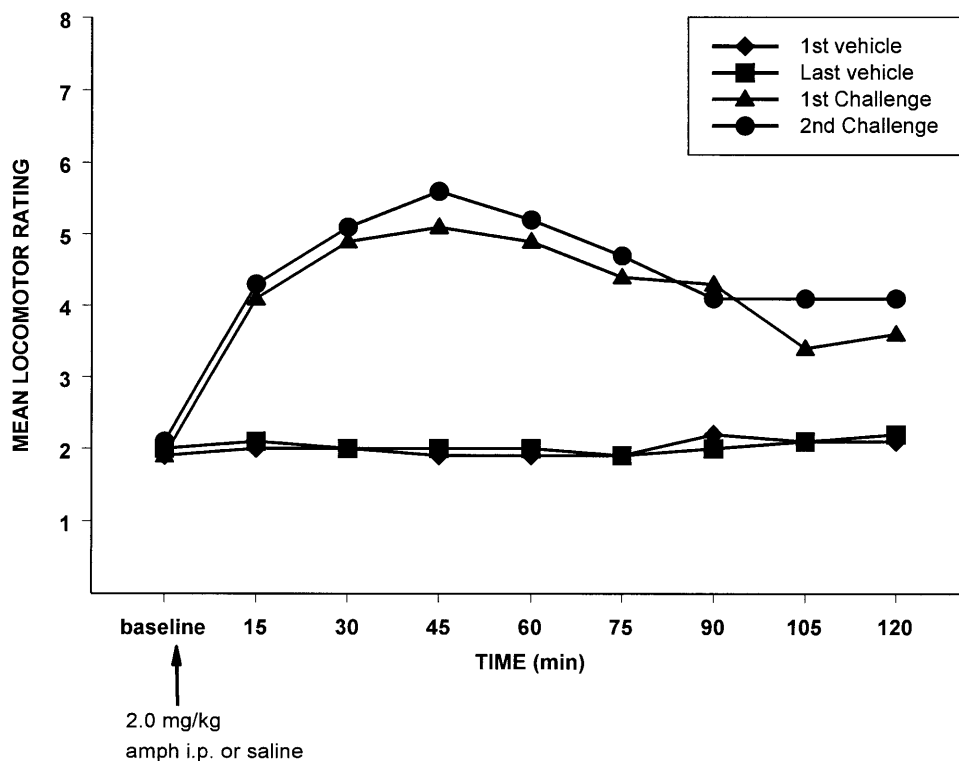
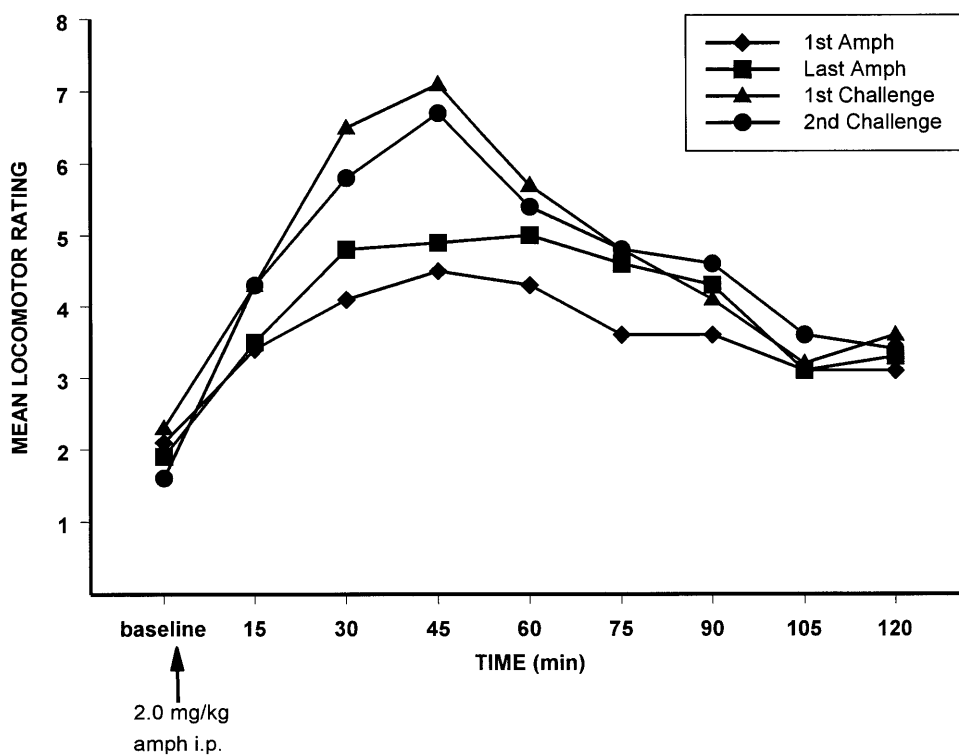


Fig. 6 Mean locomotor ratings in rats ($n=8$) pretreated with amphetamine (2.0 mg/kg i.p.) and then subsequently challenged with amphetamine (2.0 mg/kg i.p.) 10 and 19 days later. The initial two injections of amphetamine enhanced motoric activity relative to baseline levels. Moreover, activity at 45 min during the first amphetamine challenge (day 10) was significantly greater than activity at either of the two pretreatment sessions. There was a trend for activity (45 min) during the second challenge session (day 19) to be enhanced, relative to the pretreatment sessions, however this difference did not reach statistical significance



and second ($Z=-2.371$, $P=0.018$) challenge sessions. Moreover, the amphetamine-induced activity at 45 min was enhanced above the last vehicle session in both the first ($Z=-2.388$, $P=0.017$) and second ($Z=-2.371$, $P=0.018$) challenge sessions. There was, however, no difference in amphetamine-induced activity between the first and second challenge session ($Z=-1.054$, $P=0.292$).

Locomotor activity in rats pretreated with amphetamine

Figure 6 illustrates the locomotor activity during the four microdialysis sessions in rats pretreated with amphetamine. As with vehicle-pretreated rats, the animals during the baseline period of each session were generally awake but lying immobile and there were no differences

among the four dialysis sessions (all P values >0.1). Amphetamine administration increased motor activity (45 min) above basal values during the first ($Z=-2.542$, $P=0.012$) and second exposures ($Z=-2.542$, $P=0.012$) of the pretreatment regimen. There was no difference in the extent of activation (at 45 min) between the two sessions ($Z=-0.677$, $P=0.498$). The administration of amphetamine at the first withdrawal period resulted in potentiated locomotor activity (45 min, first challenge vs last pretreatment, $Z=-2.38$, $P=0.017$). Although Fig. 6 suggests that this potentiation was also evident in the second withdrawal period, this difference, relative to the final pretreatment injection, remained insignificant ($Z=-1.364$, $P=0.172$). Finally, there was no significant difference, at the 45-min timepoint, between the level of motoric activity in the first and second challenge sessions ($Z=-0.632$, $P=0.527$).

Discussion

The results of this experiment demonstrate that repeated exposure to the psychostimulant amphetamine potentiates the stimulation of cortical ACh release seen following an acute administration of the drug. As expected, the amphetamine pretreatment also sensitized the drug's effects on motoric activity. The sensitized efflux of cortical ACh was not accompanied by a similar potentiation of the effects on NAC DA efflux. The discussion below focuses on four issues surrounding the interpretation of these results: (a) methodological features of the pretreatment and microdialysis paradigm, (b) inter-relations between the dependent measures of sensitization, (c) potential neuronal mechanisms mediating the effect of repeated administration of amphetamine on cortical ACh release, and (d) implications of sensitized cortical ACh release for attentional processing and addictive drug use.

Methodological considerations

To our knowledge, this is the first experiment to simultaneously assess DA efflux in the NAC and ACh efflux in cortex in awake rats, following repeated exposure to amphetamine, using repeated microdialysis testing. The benefit of this experimental protocol is that it allows, within the same animals, the measurement of basal release data (during the first exposure to amphetamine) as well as the potential sensitization of transmitter release following different periods of withdrawal. Generally, the validation of a repeated perfusion design necessitates a randomization of treatment conditions across the dialysis sessions (that is not possible in the present experiment) and the demonstration that there are no systematic interactions between dialysis session and the experimental manipulations. We have previously used this criterion to validate the repeated perfusion technique for cortical (Moore et al. 1995b) and striatal (Johnson and Bruno 1995) ACh efflux as well as GABA efflux in striatum

and substantia nigra (Byrnes et al. 1997). In the present experiment, there was an overall decrease in basal ACh efflux between the first session and the subsequent three sessions. Importantly, however, this session effect did not interact with treatment group (vehicle- or amphetamine-pretreated). Therefore, the augmented ACh efflux seen in rats pretreated with amphetamine is unlikely to reflect some consequence of the repeated dialysis paradigm. The repeated dialysis procedure for measuring DA efflux within NAC has also been recently validated. Furthermore, the ability of TTX to suppress basal DA efflux (average reduction of 85%) was unaffected by three microdialysis sessions (Arnold unpublished observations).

Potential interactions among the dependent measures of sensitization

The potential relationships between the sensitized cortical ACh efflux and motoric activity require discussion, as several reports have demonstrated that manipulations that increase locomotor activity also increase cortical ACh efflux (Acquas et al. 1996; Day and Fibiger 1992; but see Moore et al. 1995a). These findings suggest that the sensitized cortical ACh release might have been secondary to amphetamine's more classic sensitization of locomotor activity. Differences in the onset of the effects on motor behavior and cortical ACh release suggest that this is not a sufficient explanation. In the present experiment, sensitized motor behavior was evident following the amphetamine challenge on the first withdrawal day (day 10) whereas the potentiation of cortical ACh release was not evident until the second withdrawal day (day 19).

The sensitization of cortical ACh efflux and motoric activity were not accompanied by a significant enhancement of the drug's effects on DA efflux. These negative data may seem unexpected given the general perception that amphetamine-induced behavioral sensitization is consistently accompanied by a potentiation of the drug's effects on extracellular DA in the NAC. However, a more thorough inspection of the available literature reveals the full range of changes in DA efflux following repeated exposure to amphetamine. Clearly, there are examples of sensitized NAC DA efflux following repeated drug administration (see Paulson and Robinson 1995; Paulson et al. 1991; Robinson et al. 1988; Wolf et al. 1993). However, similar to the present study, there are also reports of no potentiation of NAC DA efflux following repeated administration of amphetamine (Paulson and Robinson 1995; Wolf et al. 1993). Finally, several reports suggest that the magnitude of the stimulated NAC DA efflux is actually *attenuated* in behaviorally sensitized animals (see, for example, Segal and Kuczenski 1992). This variability in the response of NAC DA efflux undoubtedly reflects many paradigmatic differences, including constant vs escalating dose regimens, duration and spacing of pretreatment protocol, dose of challenge injection, number of drug challenges, and duration of withdrawal period (see the discussion in Paulson and Robinson 1995).

Potential neuronal mechanisms mediating the sensitization of cortical ACh efflux

The neuronal mechanisms underlying the sensitizing effects of repeated amphetamine administration on cortical ACh release are not well understood. Most studies on the neuronal mechanisms of psychostimulant sensitization have focused on the development and expression of enhanced locomotor activity. There is an extensive literature on the role of dopaminergic (Henry and White 1995; Vezina 1993; Wolf et al. 1993) and glutamatergic (Cervo and Samanin 1996; Churchill et al. 1999; Kalivas and Duffy 1998; White et al. 1995; Wolf 1998) transmission within the ventral tegmental area and NAC mediating the induction and expression of behavioral sensitization, respectively. These results, coupled with demonstrations that accumbens efferents can modulate the excitability of basal forebrain neurons (Mogenson et al. 1980), support an initial focus on altered accumbens-basal forebrain interactions in the sensitization of cortical ACh release following pretreatment with amphetamine.

A priori, insights into the neuronal mediation of amphetamine-induced sensitization of cortical ACh release may be speculated to come from studies on the effects of *acute* administration of the drug on cortical ACh release. Recently, we have directly tested the contributions of NAC DA receptors to the stimulatory effects of an *acute* systemic injection of amphetamine on cortical ACh release. In contrast to the model discussed above, intra-accumbens perfusions of D1, D2, or D1 + D2 antagonists did not attenuate the ability of amphetamine to stimulate cortical ACh release, indicating that NAC DA receptor activity is not *necessary* for the effects of amphetamine on cortical ACh. Furthermore, intra-accumbens perfusions of amphetamine, despite large increases in NAC DA levels, did not stimulate cortical ACh release, suggesting that increases in NAC DA receptor activity are not *sufficient* in mediating the effects of acute amphetamine on cortical ACh efflux.

The necessity and sufficiency of NAC DA receptor activity for the effects of *repeated* administration of amphetamine on cortical ACh release are currently being assessed. It cannot be excluded that the sensitizing effects of repeated amphetamine on cortical ACh release are not mediated via NAC dopaminergic transmission but via amphetamine-sensitive DA systems outside of the NAC or non-dopaminergic systems. In regards to the former, a single administration of amphetamine was recently found to increase DA efflux within the basal forebrain (Arnold et al. unpublished observations). Also, local DA receptors have been shown to modulate the excitability of basal forebrain neurons to iontophoretic application of glutamate and GABA (Johnson and Napier 1997). With regard to the latter speculation, studies using cocaine have documented increases in ventral tegmental (Kalivas and Duffy 1998) and NAC glutamate efflux (Pierce et al. 1996) or responsivity to glutamate (White et al. 1995; for review see Wolf 1998). Conceivably, these changes may indirectly facilitate ACh release at

the level of cortical terminals or at the cell body level within basal forebrain.

Implications of sensitized cortical ACh release for compulsive drug use

Traditionally, much of the research on the neurochemical mechanisms underlying drug addiction has focused on changes in mesoaccumbens dopaminergic transmission. This work has focused on alterations in reward processing or hedonic value and, more recently, on the role of NAC DA in the attribution of incentive salience to drug-related stimuli (Berridge and Robinson 1998; Robinson and Berridge 1993). These more recent conceptualizations of the functions of NAC DA may serve to unravel the cognitive mechanisms underlying the experience of 'reward' and craving, and consequently to determine the brain mechanisms mediating the actual information processing that leads to compulsive addictive drug use. The present data suggest that increases in cortical ACh release represent an additional neural mediator of the effects of repeated psychostimulant administration. Moreover, the extensively studied role of cortical ACh in information processing (see, for example, Sarter and Bruno 1997, 1999) makes this system a key candidate for a neuronal mechanism mediating the cognitive processes participating in addictive drug use. In subjects suffering from addiction to psychostimulants, drug-induced sensitization of cortical ACh release may, through this neuronal system's documented role in attentional processing, underlie the persistent focus on the processing of drug-associated stimuli and contexts, and the resulting exhaustion of processing resources for competing activities. Such processes profoundly decrease the likelihood that an addicted subject selects behavioral alternatives to activities focusing on addictive drug acquisition and drug use. To the extent that the hypothesis that psychostimulant-induced increases in cortical ACh mediate such a process generalizes to other addictive drugs, and to the extent that NAC DA contributes to the development of sensitized cortical ACh efflux (above), the present results may point to a neuronal mechanism that is downstream from the events in the NAC and that possibly mediates crucial aspects of the effects of addictive drugs that traditionally have been attributed to effects on NAC DA.

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