

Toward a Neuro-Cognitive Animal Model of the Cognitive Symptoms of Schizophrenia: Disruption of Cortical Cholinergic Neurotransmission Following Repeated Amphetamine Exposure in Attentional Task-Performing, but Not Non-Performing, Rats

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Impairments in attentional functions and capacities represent core aspects of the cognitive symptoms of schizophrenia. Attentional performance has been demonstrated to depend on the integrity and activity of cortical cholinergic inputs. The neurobiological, behavioral, and cognitive effects of repeated exposure to psychostimulants model important aspects of schizophrenia. In the present experiment, prefrontal acetylcholine (ACh) release was measured in attentional task-performing and non-performing rats pretreated with an escalating dosing regimen of amphetamine (AMPH) and following challenges with AMPH. In non-performing rats, pretreatment with AMPH did not affect the increases in ACh release produced by AMPH-challenges. In contrast, attentional task performance-associated increases in ACh release were attenuated in AMPH-pretreated and AMPH-challenged rats. This effect of repeated AMPH exposure on ACh release was already present before task-onset, suggesting that the loss of cognitive control that characterized these animals' performance was a result of cholinergic dysregulation. The findings indicate that the demonstration of repeated AMPH-induced dysregulation of the prefrontal cholinergic input system depends on interactions between the effects of repeated AMPH exposure and cognitive performance-associated recruitment of this neuronal system. Repeated AMPH-induced disruption of prefrontal cholinergic activity and attentional performance represents a useful model to investigate the cholinergic mechanisms contributing to the cognitive impairments of schizophrenia.

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INTRODUCTION

As reflected by the NIMH Initiative 'Measurement and Treatment Research to Improve Cognition in Schizophrenia' (MATRICS), the determination of the neuronal abnormalities that underlie the cognitive impairments of schizophrenia and the development of novel pro-cognitive treatments are pressing research objectives. Based on the fundamental role of attentional processes for learning and general cognitive control, attentional dysfunctions have been considered a central and even essential characteristic of schizophrenia (Braff, 1993; Braff and Light, 2004; Braver

et al, 1999; Keefe *et al*, 2006; McGhie and Chapman, 1961; Nuechterlein *et al*, 1994; Venables, 1964).

Attention describes the cognitive states and operations that govern the readiness for the detection of changes in the stimulus situation, the selection of such changes over irrelevant 'noise' for further processing, and the management of attentional resources for the detection and processing of competing stimuli. Substantial evidence supports a crucial role of the cortical cholinergic input system in the mediation of attentional functions and capacities (Everitt and Robbins, 1997; Sarter *et al*, 2001, 2005a). Removal of cortical cholinergic inputs produces persistent impairments in attentional performance. Furthermore, attentional performance is associated with increases in cortical acetylcholine (ACh) release that are not observed in animals performing tasks controlling for the non-attentional aspects of performance (Dalley *et al*, 2001; Himmelheber *et al*, 1997, 2000; Kozak *et al*, 2006; McGaughy *et al*, 1996, 2000; Passetti *et al*, 2000; Turchi

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and Sarter, 1997, 2000). The available evidence indicates that the cortical cholinergic input system mediates not one particular aspect of attention but supports a range of attentional abilities, including sustained, selective, and divided attention.

Although reduced muscarinic receptor densities in the cortex of schizophrenics have been reported (Crook *et al*, 2000, 2001; Hyde and Crook, 2001; Newell *et al*, 2007; Raedler *et al*, 2003), the status of cortical cholinergic neurotransmission in schizophrenia remains poorly understood. Owing in part to the lack of methods capable of assessing dynamic aspects of cholinergic dysregulation in humans, the potential contribution of cholinergic dysregulation to the cognitive symptoms of patients is unknown.

Repeated exposure to psychostimulants has long been known to produce psychotogenic effects in humans (Bell, 1965, 1973; Kokkinidis and Anisman, 1981; LeDuc and Mittleman, 1995; McDonald, 1964; O'Flanagan and Taylor, 1950; Snyder, 1973; Snyder *et al*, 1972; Wallis *et al*, 1949; Weiner, 1964). Furthermore, the effects of repeated psychostimulant exposure in healthy humans and animals model important neurobiological, behavioral, and cognitive aspects of schizophrenia (Castner and Goldman-Rakic, 1999, 2003; Kapur, 2003; Laruelle, 2000; Lieberman *et al*, 1997; Robinson and Becker, 1986; Segal *et al*, 1981; Segal and Janowski, 1978; Strakowski *et al*, 1997; Yui *et al*, 1999), including the deficits in sensorimotor gating and attentional processing (Crider *et al*, 1982; Martinez *et al*, 2005; Sarter *et al*, 2005b; Tenn *et al*, 2003). Furthermore, repeated psychostimulant exposure models the sensitization of the mesolimbic dopamine system that has been demonstrated in never-medicated patients and during psychotic periods (Abi-Dargham *et al*, 1998; Laruelle, 2000; Laruelle and Abi-Dargham, 1999; Laruelle *et al*, 1996, 1999; Strakowski *et al*, 1997). Based on evidence suggesting close links between the mesolimbic dopamine system and basal forebrain cholinergic neurons (Moore *et al*, 1999; Neigh-McCandless *et al*, 2002; Neigh *et al*, 2004; Zmarowski *et al*, 2005), abnormal regulation of the cortical cholinergic input system has been hypothesized to represent an integral component of the dysregulated forebrain systems responsible for the cognitive symptoms of schizophrenia (Sarter *et al*, 2005b). Previous findings indicating psychostimulant exposure-induced alterations in the regulation of basal forebrain cholinergic neurons are consistent with this hypothesis (Martinez *et al*, 2005; Nelson *et al*, 2000). However, this evidence does not form the basis for hypotheses describing unidirectional, causal relationships between dysregulated dopaminergic and cholinergic systems. Escalating bidirectional interactions between abnormally regulated mesolimbic projections to the basal forebrain and prefrontal cortex, and aberrant cholinergic recruitment of prefrontal neurons projecting to mesolimbic regions may ultimately be responsible for the disruption of prefrontal-mesolimbic information processing that is widely hypothesized to underlie the cognitive symptoms of schizophrenia. The present focus on the regulation of the prefrontal cholinergic input system is based on the extensive evidence linking this system to fundamental attentional processes.

The present experiment utilized an escalating dosing pretreatment regimen of amphetamine (AMPH) that is known to generate neurobiological and behavioral char-

acteristics resembling psychostimulant psychosis (Paulson *et al*, 1991; Segal and Kuczenski, 1997). This regimen produces lasting psychomotor sensitization without yielding neurotoxicity (Paulson *et al*, 1991; Paulson and Robinson, 1995; Robinson and Camp, 1987; Robinson *et al*, 1988). Importantly, psychomotor sensitization, locomotor hyperactivity, or stereotypies are not observed in attentional task-performing rats following the repeated administration of AMPH, perhaps as a result of the constraining of the animals' behavior by the operant and attentional requirements of the task (Martinez *et al*, 2005). Furthermore, it is important to note that the administration of AMPH-challenges is thought to model the role of stressors in eliciting psychotic episodes, and/or in revealing a sensitized mesolimbic system (Moghaddam, 2002; Robinson and Becker, 1986) that can trigger active disease periods (Lieberman *et al*, 1997; Ventura *et al*, 1989; Yui *et al*, 1999).

The present study was designed to determine the effects of pretreatment and challenge with AMPH on ACh release in the medial prefrontal cortex (mPFC) in animals performing a task taxing attentional capacities and in animals that did not perform a task. This experiment was guided by the general hypothesis that in order to demonstrate the abnormal regulation of a neurotransmitter system, recruitment of that system, by behavioral and cognitive operations relevant to that system, is required. The results support this hypothesis and indicate that repeated exposure to AMPH disrupts the regulation of cholinergic projections to the prefrontal cortex and thereby cognitive task control. Furthermore, these findings indicate the usefulness of experiments designed to measure the effects of repeated AMPH exposure on cognitive performance and, simultaneously, performance-associated increases in ACh release, as a model for research on the neuronal mechanisms underlying the cognitive symptoms of schizophrenia as well as on the development of novel treatments for the cognitive symptoms of this disorder.

METHODS

Animals and Animal Housing

Twenty-four male Fischer-344/Brown-Norway F1 hybrid rats (Harlan Sprague-Dawley, Indianapolis, IN), weighing between 300 and 350 g at the beginning of behavioral training, were housed individually in a temperature (23°C)- and humidity (45%)-controlled environment with a 12:12 light/dark cycle (lights on at 0700 hours). Animals were handled extensively before the beginning of training. Food (Rodent Chow, Harlan Teklad, Madison, WI) was available *ad libitum*, whereas water was available only during behavioral training as reward (below) as well as for 8 min in the home cage following daily operant training. Animal care and experimentation were performed in accordance with protocols approved by the University Committee On Use and Care of Animals of the University of Michigan (UCUCA).

Behavioral Training and Testing

Apparatus. Behavioral training was conducted using 12 operant chambers (Med-Associates, St Albans, VT), each

enclosed within a sound-attenuating compartment and equipped with three panel lights (2.8 W), two retractable levers and a water dispenser delivering 30 μ l water per reward into a cup located between the two levers. A house light (2.8 W) was located on the rear wall. Signal presentation, lever operation, water delivery, and data collection were controlled by a PC running Med-PC for Windows software (V 4.1.3; Med-Associates).

Task training. Training methods and evidence in support of the validity of performance measures in terms of reflecting sustained attention performance have been previously described (Arnold *et al*, 2003; McGaughy and Sarter, 1995; Sarter and McGaughy, 1998). It should be noted that the use of this particular task in this experiment does not necessarily imply that performance of specifically this task would uniquely activate the cortical cholinergic input system; rather, this form of attention can be more readily trained and tested in rats when compared with more complicated tasks designed to assess other aspects of attention, such as divided attention (Turchi and Sarter, 1997, 2000).

Training occurred between 08:00 and 18:30 hours 7 days a week. Animals were initially trained to lever press for water in accordance with a modified FR-1 schedule. Following at least three consecutive sessions of over 120 reinforced lever presses, animals advanced to the second stage of task acquisition. Animals were first trained to discriminate between signal (1 s illumination of the central panel light) and non-signal (no illumination) events. Two seconds following the occurrence of a signal or non-signal, both levers were extended into the operant chamber and remained active for 4 s or until a lever press occurred. During signal trials, a left lever press was scored as a hit, whereas a right lever press was scored as miss. Conversely, during non-signal trials, a right lever press was scored as a correct rejection and a left lever press was scored as a false alarm. Half of all animals were trained to acquire the task using reversed response rules. Hits and correct rejections were rewarded, whereas false alarms and misses were not. During this stage of training, incorrect responses resulted in the initiation of correction trials. During correction trials, the previous trial was repeated up to three times. If an animal continued to respond incorrectly, a forced-choice trial was initiated by presenting the correct lever only following a signal or non-signal event. Correction and forced-choice trials served to facilitate the acquisition of response rules and prevent the development of a side bias.

Once animals achieved at least three consecutive days of stable performance defined as $\geq 59\%$ correct responses to both signal- and non-signal events, they advanced to the third stage of task acquisition. Signal duration was shortened and signals were presented for 25, 50, or 500 ms. The sequence of signal duration and the occurrence of signal and non-signal trials were pseudo-randomized to yield 27 trials per signal duration and 81 non-signal trials. Correction trials and forced trials were discontinued, and the intertrial interval (ITI) was shortened from 12 ± 3 to 9 ± 3 s. As will be further detailed below, measures of performance included the relative number of hits (hits/hits + misses), calculated for each signal length, and the relative

number of correct rejections (correct rejections/correct rejections + false alarms). Once animals achieved at least 3 days of stable performance, defined as $\geq 70\%$ hits to 500 ms signals, $\geq 70\%$ correct rejections, and $\leq 50\%$ omissions to 25 ms signal), they began training in the final task (see Figure 1). House lights were illuminated throughout the session. This important final modification requires that animals constrain their behavior to maintain persistent orientation toward the intelligence panel. Each session lasted approximately 40 min. The pretreatment regimen was initiated after animals maintained criterion performance ($\geq 70\%$ hits to 500 ms signals, $\geq 70\%$ correct rejections, and $\leq 20\%$ omissions) for 3 consecutive days.

Performance measures. Measures of performance included hits, misses, correct rejections, false alarms, and omissions. The relative number of hits and correct rejections was calculated as described above. To obtain an overall index of

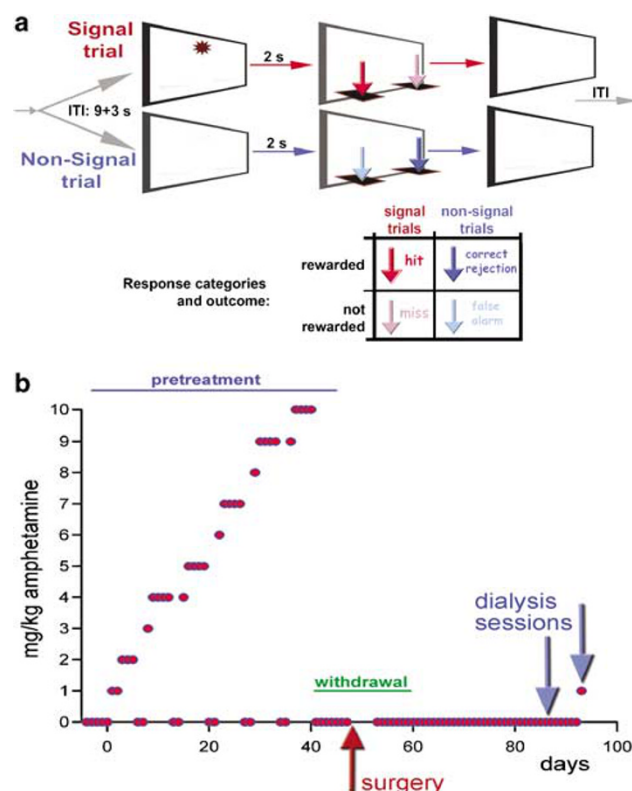


Figure 1 Top: schematic illustration of the sequences of events and the two trial types of the sustained attention task (a). A session consisted of 162 signal or non-signal trials. Correct responses in signal trials (hits) and non-signal trials (correct rejections) were rewarded (see arrows), whereas incorrect responses (misses and false alarms, respectively) were not. The ITI was variable to limit the animals' ability to time an event. Bottom: Illustration of the AMPH-pretreatment regimen and the overall timeline of main events including pretreatment, withdrawal period, surgery and the assessment of AMPH-challenges on attentional performance and performance-associated ACh release in the prefrontal cortex. Animals were treated twice a day during the pretreatment phase, before the daily training session and 8 h later (the ordinate depicts the dose that was given twice daily; each dot depicts one day and dose; see Methods for details). Control animals received vehicle throughout the pretreatment regimen (not shown) and, similar to AMPH-pretreated rats, AMPH-'challenges' following the 'withdrawal' period. Non-performing animals were treated likewise, except that the task was never activated.

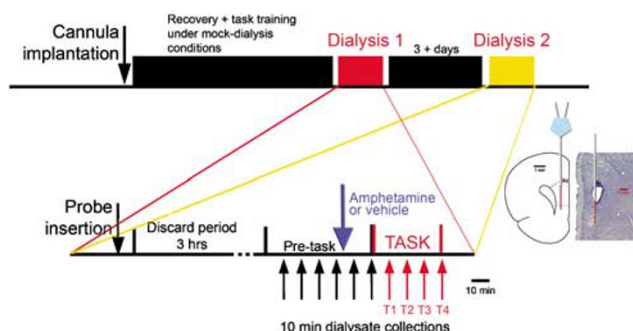


Figure 2 Main sequence of events following surgery (top) and detailed illustration of events during an individual dialysis session (lower line). As detailed in the Methods section, animals were implanted with a guide cannula for the later insertion of a microdialysis probe 7 days following the completion of the pretreatment regimen (see also Figure 1). Following a period of subsequent recovery and daily behavioral training under mock-dialysis conditions, animals underwent two sessions during which probes were inserted into the prefrontal cortex and perfused. Vehicle or AMPH-challenges were administered during these sessions. During an individual dialysis session, the probe was inserted early in the morning, the animal was placed into the operant chamber, and the probe was connected to syringes and pumps. During the next 3 h, the probe was perfused and dialysates were discarded. The three collections before drug administration were used to determine the stability of ACh efflux and basal ACh efflux. Following two collections after the administration of drug or vehicle, the task was activated and four 10-min samples were collected. The inset shows a representative placement of a microdialysis probe in the prelimbic region (PrL), superimposed over a schematic section (left) and an actual coronal section (probe length reflects approximately the 1 mm scales inserted in both the schematic and the photomicrograph).

performance that reflects the performance in trials involving signals as well as non-signal events, the vigilance index (VI) was calculated based on the relative number of hits (h) and false alarms (fa) using the formula: $VI = (h - fa) / [2(h + fa) - (h + fa)^2]$. This index is comparable to the sensitivity index (SI) (Frey and Colliver, 1973) except that VI is based on the relative number of hits and false alarms, whereas SI is calculated using the probabilities for hits and false alarms. Thus, VI values are not confounded by errors of omission. Values for VI can vary from +1.0 to -1.0, with +1.0 indicating that all responses were scored as hits or correct rejections, 0 indicating an inability to discriminate between signal and non-signal events, and -1 indicating that all responses to signals were misses and all responses to non-signals were false alarms. The index was calculated for each signal duration ($VI_{500,50,25}$). Finally, errors of omission were recorded. Performance measures were calculated collectively for the entire session as well as separately for each of four task blocks (10 min each; see Figure 2).

Pretreatment Regimen and Challenges

After reaching performance criterion, animals were randomly divided into two groups ($n = 7$ each) designated to be pretreated with AMPH or vehicle (saline; SAL). Animals were administered either *d*-AMPH sulfate (1–10 mg/kg; i.p.; concentrations included salt weight; dissolved in 1.0 ml/kg of 0.9% saline; Sigma, St Louis, MO) or saline (1.0 ml/kg i.p.) twice per day, with approximately 8 h separating the two injections (Figure 1). Animals received the first injection at approximately 0800 hours and were placed

immediately into the test chambers. Task onset was 20 min post-injection. Following the completion of the test session, animals were promptly returned to their home cages. The second dose of drug or vehicle was given in the home cage at approximately 16:00 hours. AMPH doses were administered in elevating increments over the course of 40 days (Paulson *et al*, 1991). All animals were given injections of saline on weekends to mimic the 'runs and crashes' known to foster psychostimulant-induced psychosis (see Figure 1; for references see Introduction). Following AMPH doses of 2 mg/kg or higher, task performance was disrupted and animals omitted all trials. In order to control for the potentially confounding effects of substantially lower amounts of task practice in AMPH-treated animals, animals treated with vehicle were placed in the testing chambers but not allowed to perform for an equivalent number of sessions during weekdays, whereas AMPH-treated animals received doses > 2 mg/kg (Martinez *et al*, 2005). Both saline- and AMPH-treated animals performed the task on weekends.

In our previous experiment employing this AMPH regimen in task-performing animals (Martinez *et al*, 2005), we found that following termination of the pretreatment regimen, AMPH-treated animals' performance required about 2 weeks of continued training to return to baseline, reflecting the general behavioral depression observed during this period of withdrawal (Paulson *et al*, 1991). As this experiment was not designed to study aspects of withdrawal, and in order to implant guide cannula (for the later insertion of microdialysis probes) relatively close to the actual microdialysis test sessions, surgery was conducted 7 days into this withdrawal period. The daily testing continued until surgery (below) and resumed following 5 days of post-surgery recovery during which food and water were available *ad libitum*. All subsequent testing was conducted in operant chambers modified to accommodate the procedures for microdialysis (see below).

The effects of AMPH-challenges were determined following the administration of 1.0 mg/kg of AMPH. This dose is the first dose given during the pretreatment regimen and was observed earlier not to produce acute effects on attentional performance (Martinez *et al*, 2005). Likewise, this dose did not affect the performance of saline-pretreated animals when given as a 'challenge' (Martinez *et al*, 2005). Therefore, significant differences between the effects of 1.0 mg/kg when given as a challenge to animals pretreated with AMPH or vehicle can be attributed to the differential pretreatment history. Thus, final comparisons were based on data from animals pretreated with SAL or AMPH and challenged with either SAL or AMPH, resulting in four treatment conditions and groups (SAL/SAL; AMPH/SAL; SAL/AMPH; AMPH/AMPH).

Non-Performing Rats

Non-performing animals ($n = 10$) were handled extensively using procedures identical to task-performing rats, including the daily transport between home cages and operant chambers and the number and the timing of injections of AMPH ($n = 6$) or SAL ($n = 4$). However, the task was never activated for these animals and, as water reward was not delivered, they were not water deprived. Similar to task-performing animals, non-performing animals underwent

stereotaxic surgery 7 days following the cessation of pretreatment. Animals were allowed to recover for 5 days and then resumed the handling procedures for the remainder of the withdrawal period. The effects of AMPH- or saline-challenges on ACh release were determined 33.6 ± 6.4 days (M; SEM) after completion of the pretreatment period (in order to match the interval that was required for performing rats; see below).

Surgical Methods

Surgery was performed under aseptic conditions. Initial anesthesia was induced with 4–5% isoflurane by placing the animal in an anesthetic chamber (Anesco/Surgivet, Waukeasha, WI). Gas was carried via oxygen at a flow rate of 0.6 ml/min. Animals were also given a preoperative injection of an antibiotic (Amikacin, 100 mg/kg; s.c.). Heads were shaved using electric clippers and cleaned with 70% ethanol and iodine tincture. Ophthalmic ointment was applied to lubricate the eyes. Animals were then mounted into a stereotaxic instrument (David Kopf, Tujunga, CA). Isoflurane was administered via a face mask at 1.5–2% for the duration of surgery. Microdialysis guide cannula (0.38 mm o.d.; Sci Pro, Sanborn, NY, USA) were implanted dorsal to the prelimbic region of the right hemisphere at the following coordinates: AP (from Bregma: 2.9 mm, ML: 0.6 mm, DV: 0.6 mm (from dura). After surgery, rats were returned to their home cages and allowed to recover for 5 days with free access to food and water. Thereafter, the water deprivation schedule resumed and animals were returned to behavioral training until they regained performance criterion (if applicable). Before daily test sessions, the dummy stylets were removed and polyethylene tubing was attached in order to habituate the animals to performing while being dialyzed.

Microdialysis Methods

Following recovery, animals resumed operant training in chambers modified to accommodate microdialysis procedures. The modified operant chambers used to measure ACh release in task-performing rats featured a taller recessed water delivery area (9.0×5.0 cm, height \times width) to allow access for animals with a probe inserted and inlets and outlets attached, and to accommodate the liquid swivels, syringes and pumps outside the chambers. This was performed in order to allow collection of dialysates outside of the chambers without interfering with the animals' ongoing performance.

Furthermore, the length of the test sessions was set to 40 min to correspond exactly with the timing of four dialysate collections (10 min each). Procedures designed to foster habituation to microdialysis procedures, particularly the tethering during task performance, were initiated at this point. Because of the subsequent requirement for extended pretask microdialysis discard periods (3 h), the collection of four baseline dialysates, and an additional two dialysates following drug treatment and before task onset (see Figure 2), rats were placed in the operant chambers 240 min before task onset. The houselight was illuminated for the entire time the animals were in the operant chambers.

After being transferred to the modified operant chambers, animals were retrained to a performance criterion ($\geq 60\%$ hits to 500 ms signals, $\geq 65\%$ correct rejections, and $\leq 20\%$ omissions for three consecutive sessions). This criterion was more lenient than for the original acquisition (above), because the performance of tethered animals was more variable and slightly impaired relative to the performance of non-tethered animals. As these animals required a relatively large number of sessions to meet criterion performance, on average 34.7 ± 3.7 days elapsed between completion of the pretreatment period and the first microdialysis session. Animals were dialyzed at least twice, first following the administration of saline and 6 ± 1 days later following the administration of AMPH. Considering evidence suggesting that even a single dose of AMPH produces sensitizing effects (Vanderschuren *et al*, 1999), the effects of saline-‘challenges’ were always tested first. In the event that dialysis sessions preceded by saline administration did not result in detectable levels of ACh as a result of probe failure or severed tubing, a maximum of two additional sessions were conducted in order to generate a complete data set for each animal. Repeated insertion of probes over a period of weeks, up to four insertions, was repeatedly demonstrated to generate similar basal ACh release levels (see Results) sensitive to the blocking of voltage-regulated sodium channels with tetrodotoxin (Bruno *et al*, 1999; Moore *et al*, 1993, 1995a,b, 1996).

Before insertion of a concentric microdialysis probe into the brain (Model MAB4; membrane o.d.: 0.24 mm; membrane length: 3.0 mm; Sci Pro), probe recovery was determined *in vitro* by placing the probe into a 1.0 pmol ACh solution and collecting for 10 min. Probes that were used exhibited recoveries of $\geq 9\%$. Probes were perfused at a rate of 2.0 μ l/min with artificial cerebrospinal fluid, pH 6.9 ± 0.1 , containing the following (in mM): 126.5 NaCl, 27.5 NaHCO₃, 2.4 KCl, 0.5 Na₂SO₄, 0.5 KH₂PO₄, 1.2 CaCl₂, 0.8 MgCl₂, and 5.0 glucose. Note that the perfusion medium did not contain an acetylcholinesterase inhibitor.

Dialysate collections were frozen at -80°C until ACh contents were determined using high performance liquid chromatography coupled with electrochemical detection (ESA, Chelmsford, MA), using a mobile phase containing 50 mM sodium phosphate. ACh was separated from choline on UniJet microbore analytical column (Bioanalytical Systems Inc. (BASi), West Lafayette, IN) and catalyzed on a post-column solid-phase reactor containing acetylcholinesterase and choline oxidase. ACh was hydrolyzed to acetate and choline, and choline oxidized to hydrogen peroxide and betaine. The amount of hydrogen peroxide corresponding to ACh was then detected using a ‘peroxidase-wired’ glassy carbon electrode with an applied potential of -200 mV (Huang *et al*, 1995). The concentration of ACh was calculated by integrating the area under the peak and fitting this value to a regression line containing values of ACh that were in the expected range of the *in vivo* dialysates. The detection limit of this system averaged 2 fmol/15 μ l.

Histological Verification of Probe Placements

Within 1 week following the last microdialysis session, animals were given an overdose of sodium pentobarbital

and underwent cardiac perfusion with 0.1 M of phosphate buffer followed by 4% buffered formalin. The brains were post-fixed in formalin overnight, and transferred to a 30% sucrose phosphate buffer solution. Sections (40 μ m thick) surrounding the probe and cannula sites were mounted, stained with cresyl violet, and examined for probe placements.

Statistical Methods

Statistical analyses for performance and dialysis data were conducted using mixed model analysis of variance (ANOVAs). As basal ACh release data did not differ between groups (see Results), the effects of the challenges on ACh release in performing and non-performing rats were conducted based on values expressed as the percent change values from mean basal ACh release (average of the last three collections before the administration of AMPH or saline). ACh release values were not corrected for probe recovery. To determine drug-induced changes in ACh release in performing rats, a mixed ANOVA on the effects of task (eg two post-drug/pre-task collections vs four task-associated collections), pretreatment (AMPH or saline), and session (AMPH- or saline-challenge) was conducted and followed, where appropriate by two- and one-way ANOVAs and multiple comparisons. Task performance was analyzed on the basis of overall performance as indicated by VI (see above for calculation). In addition, the numbers of errors of omission were analyzed. The ANOVAs determined the effects of pretreatment (AMPH vs saline), session (AMPH- or saline-challenge), and signal duration (500–25 ms) on VI and omissions. Significant main effects and interactions were followed by two- and one-way ANOVAs and Fisher's least significant difference test (LSD) for multiple comparisons. Exact *p*-values are reported for significant results, as was recommended earlier (Greenwald *et al*, 1996). Statistical analyses were performed using SPSS Version 14.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Histological Findings

As illustrated in the inset in Figure 2, dialysis probes were placed into the middle layers of the prelimbic region. In most cases, the active membrane extended either dorsally into the anterior cingulate cortex or ventrally into the infralimbic region. Neither baseline release values nor performance- and/or AMPH-induced changes in ACh release systematically differed between these minor variations in placement, and thus evidence obtained from all these placements was used for final analysis.

Effects of Repeated AMPH on ACh Release in Non-Performing Rats

Basal ACh release did not differ between non-performing animals pretreated with AMPH and vehicle ($F(1,8) = 0.085$; $p > 0.05$; Figure 3), nor did basal values differ between session (before vehicle- 'challenge' or AMPH-challenge; $F(1,8) = 0.006$; $p > 0.05$), and the two variables did not interact significantly ($F(1,8) = 0.09$; $p > 0.05$). Basal ACh

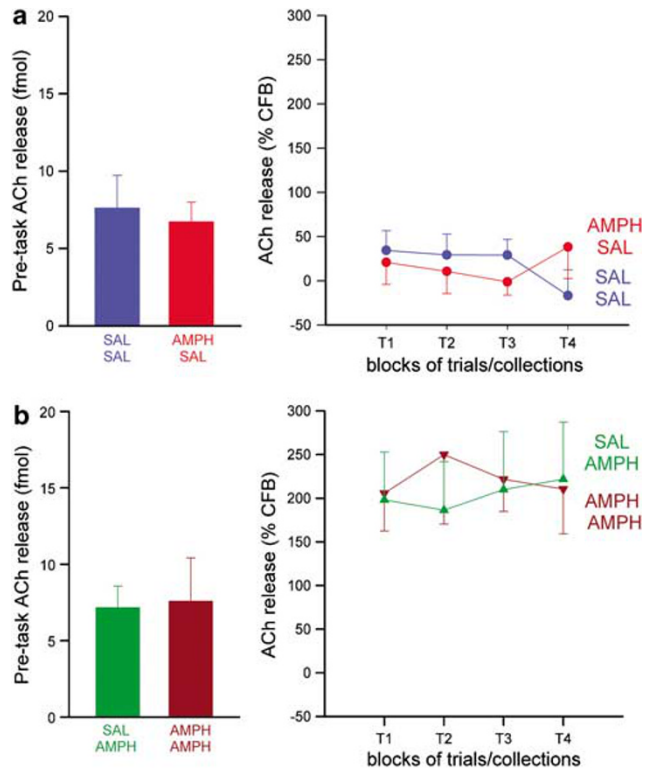


Figure 3 Effects of AMPH-pretreatment and AMPH-challenge on mPFC ACh release in non-performing rats (M; SEM). The bar graphs indicate absolute release levels (in fmol/15 μ l) before the administration of saline (SAL) or AMPH. Following saline- 'challenges', ACh release levels did not differ between saline- and AMPH-pretreated animals (a; see Results for statistical findings). AMPH-challenges resulted in comparable increases in ACh release in animals pretreated with SAL or AMPH (b). Thus, in non-performing animals, the type of pretreatment did not modify the effects of the acute challenges on ACh release.

release was 7.26 ± 1.09 fmol/15 μ l. Because of the absence of pretreatment effects on basal ACh release, the effects of vehicle- or AMPH-challenge on ACh release were expressed as percent change from baseline.

Compared with the administration of vehicle, the AMPH-challenge resulted in a significant increase in ACh release in both saline and AMPH-pretreated rats (main effect of session: $F(1,8) = 28.28$; $p = 0.001$). The increase in ACh release that resulted from AMPH-challenge did not differ between animals pretreated with vehicle or AMPH (pretreatment: $F(1,8) = 0.07$; $p > 0.05$; pretreatment \times session: $F(1,8) = 0.11$; $p > 0.05$). Furthermore, ACh release did not vary over the four collections (T1–T4 in Figure 3), and this variable did not interact with pretreatment or session (all $p > 0.05$). As illustrated in Figure 3, following saline- 'challenges', ACh release was $18.06 \pm 15.22\%$ over baseline (averaged over T1–T4); these values did not differ significantly from baseline values ($F(1,8) = 0.42$; $p > 0.05$). The AMPH-challenges resulted in an increase of $210.66 \pm 35.44\%$ over baseline ($F(1,8) = 35.13$; $p < 0.001$). *Post hoc* analyses indicated that AMPH significantly increased ACh release in both groups of animals when compared to the effects of saline- 'challenges' (both $p < 0.02$), and that neither the effects of saline- nor AMPH-challenges differed between animals pretreated with

saline vs AMPH (both $p > 0.05$). Thus, the pretreatment history of non-performing animals did not influence the AMPH-challenge-induced increases in ACh release.

Effects of Repeated AMPH on ACh Release in Performing Rats

Basal ACh release did not differ between animals pretreated with vehicle or AMPH ($F(1,12) = 2.23$; $p > 0.05$; Figure 4). Likewise, basal release did not differ before the administration of an AMPH-challenge or vehicle ($F(1,12) = 1.63$; $p > 0.05$) and the two factors (group, session) did not interact significantly ($F(1,12) = 1.62$; $p > 0.05$). Basal ACh release was 7.53 ± 1.58 fmol/15 μ l. Basal release did not differ between non-performing (above) and attentional task-performing rats ($F(1,22) = 0.00$; $p > 0.05$).

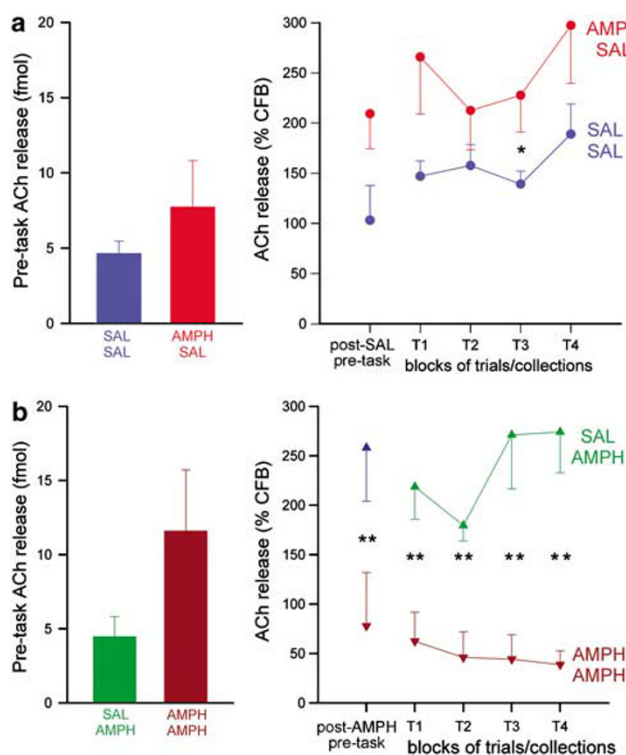


Figure 4 In attentional task-performing rats, the type of pretreatment (saline vs AMPH) determined the effects of the challenge (saline vs AMPH) on ACh release (M; SEM). The bar graphs indicate absolute release levels (in fmol/15 μ l) before the administration of saline (SAL) (a) or AMPH (b). Absolute levels of ACh release did not differ significantly between the groups or session, and they did not differ from animals that did not perform the task (Figure 3; see Results for statistical findings). AMPH-pretreatment and AMPH-challenge (AMPH/AMPH) resulted in a highly robust attenuation of performance-associated increase in ACh release (b). This effect was already present during the two collections taken before task onset, suggesting that continuous expectation of task onset and performance and/or the context of performance are sufficient to reveal the interactions between pretreatment and challenge. This observation also rejects the possibility that the attenuation of performance-associated increases in ACh release in AMPH-pretreated and -challenged animals represented merely secondary effects of the disruption of performance (* $p < 0.05$; ** $p < 0.005$, significant differences between animals pretreated with saline vs AMPH; see Results for ANOVAs).

Effects of AMPH-challenges on performance-associated ACh release. As illustrated in Figure 2, following the administration of drug or vehicle as challenges, two dialysates were collected before task onset. An overall ANOVA on the effects of task-stage (pre-task vs task), pretreatment, and challenge revealed a significant interaction between the effects of pretreatment and challenge ($F(1,12) = 31.63$; $p < 0.001$). However, there was no effect of task-stage and no interactions between task-stage, pretreatment or challenge (all $p > 0.05$). Thus, the effects of repeated AMPH exposure and AMPH-challenge on ACh release did not differ between the two collections taken before task onset and the four collections taken during task performance (see Figure 4).

Similar to the results from previous experiments (Arnold *et al*, 2002; Himmelheber *et al*, 2000; Kozak *et al*, 2006), performance of the attention task increased ACh release in the mPFC over baseline. In SAL/SAL animals, ACh release increased by $158.28 \pm 18.49\%$ over basal ACh levels during the performance of the task ($F(1,6) = 66.98$; $p < 0.001$; see Figure 4). The performance-associated increase in mPFC ACh release is comparable to the increase observed previously in animals performing this task and using similar microdialysis conditions, including the absence of an acetylcholinesterase inhibitor (Kozak *et al*, 2006).

The effects of the AMPH-challenges differed significantly between groups of rats pretreated with SAL vs AMPH (pretreatment \times challenge: $F(1,12) = 30.74$; $p < 0.0001$; main effect of challenge: $F(1,12) = 6.14$; $p = 0.03$; main effect of pretreatment: $F(1,12) = 3.00$; $p > 0.05$; the factor time (T1–T4) did not produce a main effect and did not interact with group and session; all $p > 0.05$). Figure 4 illustrates that AMPH-challenges in animals pretreated with AMPH (AMPH/AMPH) resulted in the attenuation of performance-associated increases in ACh release. Several *post hoc* comparisons further substantiated this result.

The acute administration of AMPH in SAL-pretreated rats did not affect the elevated levels of ACh release observed in animals performing this task (SAL/SAL vs SAL/AMPH; $F(1,6) = 3.88$; $p > 0.05$). In contrast, the acute administration of, or the challenge with, AMPH in animals that were pretreated also with AMPH resulted in a significant attenuation of ACh release levels when compared with animals pretreated with SAL (SAL/AMPH vs AMPH/AMPH ($F(1,12) = 29.62$; $p < 0.0001$). Multiple comparisons indicated that all data points (T1–T4) differed significantly by pretreatment (all $p < 0.004$; Figure 4, lower graph). Averaged over all time points, performance-associated ACh release in SAL/AMPH animals was $235.93 \pm 27.47\%$ over pretask baseline and $47.89 \pm 24.42\%$ in AMPH/AMPH rats. The attenuation of ACh release levels in AMPH/AMPH animals was also revealed by the within-subject comparison (AMPH/SAL vs AMPH/AMPH; $F(1,6) = 40.83$; $p = 0.001$).

The attenuated levels of ACh release observed in performing AMPH/AMPH rats did not differ significantly from release levels measured at baseline (before task onset and before drug treatment ($F(1,6) = 2.65$; $p > 0.05$). Moreover, a *post hoc* comparison between ACh release levels in AMPH/AMPH animals over all three phases (baseline, post-drug/pre-task, during performance) indicated that ACh release levels in these animals never changed from baseline ($F(2,12) = 0.79$; $p > 0.05$).

Finally, in animals pretreated with AMPH and dialyzed following vehicle-‘challenge’ (AMPH/SAL; Figure 4), performance-associated ACh release was significantly higher than in animals pretreated and ‘challenged’ with vehicle (SAL/SAL; $F(1,12) = 5.34$; $p = 0.04$; averaged over all time points: saline-pretreated: $158.28 \pm 18.49\%$ over baseline; AMPH-pretreated: $250.92 \pm 18.44\%$). Multiple comparisons indicated that release during T3 was significantly higher in animals pretreated with AMPH ($F(1,12) = 5.20$; $p = 0.042$; the effect neared significance during T1; $F(1,12) = 4.10$, $p = 0.06$; Figure 4, top graph).

Effects of Repeated AMPH on Performance

Baseline performance before the administration of challenges. During the pretreatment period, administration of escalating doses of AMPH (Figure 1b) increasingly disrupted the animals’ performance. During drug-free weekends, performance partially recovered. The pattern of the performance during the 40-day AMPH-pretreatment period and during the subsequent 2-week withdrawal period corresponded with the evidence described previously (Martinez *et al*, 2005). Before the challenge with AMPH or vehicle, the performance of all animals as measured by VI remained impaired relative to the pretreatment baseline ($F(1,12) = 5.05$; $p = 0.04$; pretreatment baseline, VI averaged over all signal durations: 0.24 ± 0.03 ; prechallenge baseline: 0.12 ± 0.03 ; see Figure 1 for timeline and Methods for additional details). Importantly, the performance of AMPH-pretreated rats did not differ from saline-pretreated rats before the administration of the challenges ($F(1,12) = 0.01$; $p > 0.05$), confirming that the relatively low level of performance at this point was not a result of AMPH-pretreatment but of the testing conditions, particularly the tethering procedures required to conduct microdialysis in task-performing animals. The number of trials omitted remained low and did not differ from pretreatment baseline levels ($F(1,12) = 0.02$; $p > 0.05$; pretreatment baseline: $12.1 \pm 6.0\%$ trials omitted/session; prechallenge baseline: $9.0 \pm 1.8\%$).

Performance following AMPH-challenges. The analysis of the effects of AMPH-challenges on performance (VI) indicated a significant interaction between the effects of pretreatment, session and signal duration ($F(2,24) = 5.27$; $p = 0.01$). Figure 5 depicts VI scores individually for the four treatment conditions and each signal duration. *Post hoc* analyses were conducted to identify the nature of this interaction. First, as was expected, the administration of AMPH as a challenge in SAL-pretreated rats (SAL/AMPH) did not affect VI (SAL/SAL vs SAL/AMPH; $F(1,6) = 0.39$; $p > 0.05$). Likewise, pretreatment with AMPH alone did not affect performance (SAL/SAL vs AMPH/SAL; $F(1,12) = 0.28$; $p > 0.05$). In contrast, the AMPH-challenge in AMPH-pretreated rats resulted in a significant decrease in performance compared with the administration of SAL in AMPH-pretreated rats (AMPH/SAL vs AMPH/AMPH; $F(1,6) = 7.50$; $p = 0.03$). Thus, interactions between the effects of pretreatment and challenge with AMPH were responsible for the disruption of performance.

Figure 5 also illustrates the role of signal duration as a factor in the significant overall interaction. One-way

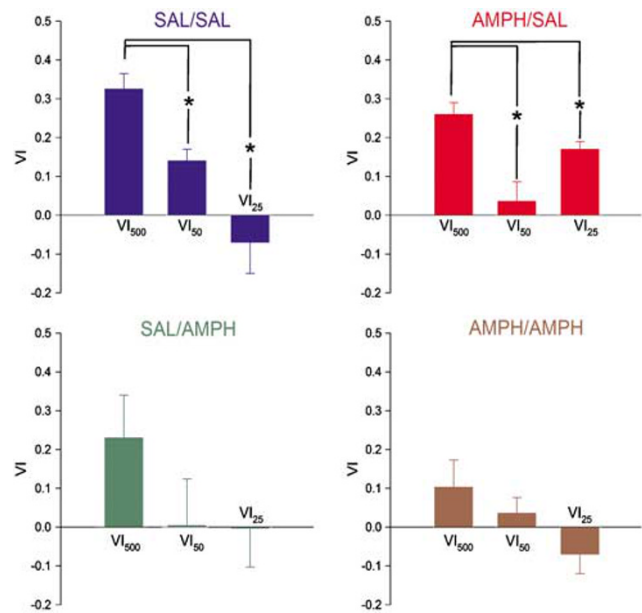


Figure 5 Effects of AMPH-pretreatment and AMPH-challenge on overall attentional performance as indicated by the VI (M; SEM). As a result of performing under dialysis conditions, including the tethering of the animals, performance was variable and levels of performance were relatively low when compared with the performance before surgery. However, AMPH-challenges resulted in significantly lower levels of performance in AMPH-pretreated animals when compared to the effects of AMPH in saline-pretreated rats. Furthermore, in contrast to SAL/SAL and AMPH/SAL rats (* $p < 0.05$; multiple comparisons using LSD tests and conducted on the basis of significant ANOVAs), the depressed levels of performance of AMPH/AMPH animals did not depend on signal duration. The performance in SAL/AMPH animals appeared to remain dependent on signal duration; however, data variability prevented statistical significance ($p = 0.07$).

ANOVAs indicated significant effects of signal duration on performance in SAL/SAL ($F(2,12) = 16.51$; $p = 0.003$) and AMPH/SAL rats ($F(2,12) = 9.33$; $p = 0.004$; see Figure 5 for multiple comparisons). As indicated in Figure 5, in SAL/AMPH animals, data variability prevented the demonstration of a significant effect of signal duration on VI ($F(2,12) = 3.48$; $p = 0.07$), whereas the performance of AMPH/AMPH rats was depressed and varied between $+0.1$ and -0.1 for all signal durations ($F(2,12) = 2.92$; $p > 0.05$). Thus, pretreatment and challenge with AMPH abolished signal duration-dependent performance; this effect is reflected in the overall significant interaction between the effects of pretreatment, challenge, and signal duration reported above.

A similar interaction between these three factors was found in the analysis of hits ($F(2,24) = 4.41$; $p = 0.02$), but not correction rejections ($F(1,12) = 1.11$; $p = 0.31$), indicating that the effects on VI were largely due to effects of the animals’ ability to detect signals. This finding is consistent with the selective impairment in signal trial performance observed following removal of the cortical cholinergic input system (McGaughy *et al*, 1996).

Finally, AMPH-challenges did not affect the animals’ errors of omission ($F(1,12) = 1.57$; $p = 0.24$). Omission rates remained generally low (5.54 ± 0.76 omissions/session, averaged over all four groups and both test sessions).

DISCUSSION

The present results indicate that in animals habituated to testing and microdialysis conditions but that did not perform the attentional task, pretreatment with an escalating dosing regimen of AMPH did not alter the effects of AMPH-challenges on prefrontal ACh release. In contrast, in rats performing the attentional task, such a challenge profoundly attenuated the increases in ACh release normally observed in animals performing this task—but only in animals that were pretreated with AMPH. Furthermore, task performance was disrupted in AMPH/AMPH animals. Based on post-drug and pretask ACh release values, the disruption of performance in AMPH/AMPH animals is concluded to represent a result of the attenuation of prefrontal cholinergic activity. These results suggest that the effects of repeated AMPH exposure on the regulation of cholinergic neurotransmission in the mPFC depend on the level of recruitment of the cholinergic system. Below, empirical limitations and interpretational complexities will be discussed, followed by an evaluation of the significance of these findings for research on the neurobiology of sensitized cognitive impairments and animal models of schizophrenia.

The present results have implications for the understanding of the relationships between ACh release and levels of attentional performance. Previous experiments indicated that increases in the demands on attentional performance, resulting from long-task periods or pharmacological challenges on performance, as opposed to increases in performance levels, correlate with increases in cortical ACh release in task-performing animals (Kozak *et al*, 2006; Passetti *et al*, 2000). This evidence corresponds with the hypothesis that increases in mPFC ACh release above normal performance-associated levels mediate the recruitment of the 'anterior attention system' and the resulting implementation of top-down mechanisms that counteract the performance decrements triggered by challenging conditions (Sarter *et al*, 2006). Based on this hypothesis, the augmented increases in ACh release observed in AMPH/SAL rats, when compared with SAL/SAL animals, may reflect the greater demands on attentional effort required to maintain normal attentional performance. Thus, AMPH/SAL animals were able to perform at control levels but required abnormally high levels of mPFC cholinergic activity to maintain performance.

The present evidence suggests that AMPH-pretreatment and AMPH-challenge disrupts attentional performance-associated increases in mPFC ACh release and therefore impaired the animals' ability to employ information concerning the presence or absence of a signal to guide the selection and execution of a response. The finding that following saline or AMPH-challenges, pretask ACh release levels did not differ significantly from ACh release levels during task performance suggests that continuous task performance, the expectation of performance and task onset, and/or being placed in the performance context, are sufficient to reveal the consequences of AMPH-pretreatment. Moreover, this finding supports the view that following repeated AMPH exposure in task-performing rats, AMPH-challenges disrupt the normal recruitment of cholinergic inputs to the PFC and therefore results in the

loss of cognitive control. In contrast, the results do not support the alternative view that repeated AMPH, via unknown mechanisms, abolished cognitive task control and that the low levels of ACh release were merely secondary to the behavioral/cognitive effects of repeated AMPH exposure.

Previous studies demonstrated that the performance of operant schedules not involving explicit demands on attention do not produce significant increases in cortical ACh release, or produce increases that are substantially lower than those associated with attentional performance. For example, cortical ACh release in rats performing a fixed interval 9 s schedule of reinforcement increased only by about 50% over baseline, despite a lever-pressing rate that was almost 10-fold the rate observed in sustained attention task-performing rats (Arnold *et al*, 2002). Likewise, operant procedures controlling for the effects of reward rate and the sensory effects of stimuli indicated that these variables do not account for the increases in ACh release observed in attentional task-performing rats (Dalley *et al*, 2001; Himmelheber *et al*, 1997). As the performance in non-cognitive procedures does not yield the levels of cholinergic activity observed in attentional task-performing animals, the interactions between recruitment of the cholinergic system and repeated psychostimulant exposure demonstrated in the present experiment would not be expected in animals performing tasks that do not tax cognitive functions.

In AMPH/AMPH animals, VI scores varied around zero across all signal durations (Figure 5). A VI score of zero indicates a loss of the ability to discriminate between signal and non-signal trials. That is, responses in signal and non-signal trials reached chance level and the animals' performance was no longer controlled by the presence or absence of a signal. Therefore, the performance of these animals no longer involved attentional processes and the processing of stimulus-response rules. Levels of ACh release in AMPH/AMPH animals performing the attention task (present experiment) were similar to ACh release levels observed in rats performing simple operant procedures not involving cognitive operations (references above). This observation is consistent with the conclusion that in AMPH/AMPH rats, cognitive task control was abolished. Indeed, ACh release in AMPH/AMPH rats did not increase significantly beyond pretask baseline. Thus, both the behavioral data and the ACh release levels support the conclusion that as a result of the pretreatment and challenge with AMPH, the animals' ability to utilize the presence or absence of signals to guide the responses was drastically impaired or, in other words, cognitive task control was disrupted.

As the pretreatment history of the animals did not modulate the effects of AMPH-challenges in non-performing animals, the present evidence provides a rather stark illustration of the view that the effective demonstration of abnormal regulation of a neuronal system requires recruitment of this system by, for example, demands on relevant behavioral or cognitive functions. This view contrasts with the widespread practice of assessing drug effects on neurotransmitter release, or with the status of neurotransmitter systems in animal models, in animals that remain passive or even anesthetized, and in the absence of

recruitment of the neuronal system of interest (Sarter *et al*, 2007).

The mechanisms underlying such drastically different modulation of the cortical cholinergic input system in response to repeated AMPH exposure remain a subject of speculation. In non-performing but extensively habituated animals, repeated AMPH-induced increases in ACh release could reflect a purely pharmacological effect, due primarily to the release of norepinephrine (Rothman *et al*, 2001; Vanderschuren *et al*, 2003) and dopamine (Robinson *et al*, 1988), both of which are capable of stimulating cholinergic neurons in the basal forebrain (Arnold *et al*, 2001; Berntson *et al*, 2003a,b; Knox *et al*, 2004; Momiyama and Sim, 1996; Napier *et al*, 1991).

In contrast, the recruitment of prefrontal cholinergic inputs in task-performing animals is thought to be mediated via direct prefrontal projections to the basal forebrain as well as via multi-synaptic circuits involving the nucleus accumbens and perhaps also the amygdala (Holland *et al*, 2000; Lee *et al*, 2006; Neigh-McCandless *et al*, 2002; Sarter *et al*, 2001, 2005a; Zaborszky, 1997, 2002; Zahm, 2000). Together with evidence demonstrating the disruption of the prefrontal modulation of accumbens neurons following repeated psychostimulant exposure (Goto and Grace, 2005), the present findings suggest that repeated AMPH-exposure disrupts such telencephalic innervation of basal forebrain cholinergic neurons and thus, prefrontal ACh release. A range of neuronal mechanisms could be responsible for such a disruption, including abnormalities in glutamatergic and dopaminergic neurotransmission in prefrontal and mesolimbic regions (Giorgetti *et al*, 2001; Lu and Wolf, 1999; Peterson *et al*, 2006; Prasad *et al*, 1995) as well as structural reorganization of prefrontal and mesolimbic neurons (Crombag *et al*, 2005; Robinson and Kolb, 2004).

As the administration of AMPH as a challenge was necessary to reveal the cholinergic and cognitive consequences of the pretreatment with AMPH, it can be speculated that these consequences were a result of interactions between increases in noradrenergic and dopaminergic neurotransmission and the recruitment of the cholinergic system by cognitive task performance. Importantly, this conclusion does not imply that the dysregulatory consequences of repeated psychostimulant exposure remain restricted to monoaminergic systems; rather, the current results indicate that increases in noradrenergic/dopaminergic systems are necessary to reveal the cholinergic and cognitive consequences of prior psychostimulant exposure. As discussed earlier (Sarter *et al*, 2005b), the administration of such challenges is thought to model the role of stressors in the initiation of active disease periods. Therefore, the cholinergic dysregulation and disruption of cognitive performance following the pretreatment and challenge with AMPH may primarily serve as a model of the active and deteriorative disease stage (Lieberman *et al*, 1997). In addition, however, the results indicate that AMPH-pretreatment alone (AMPH/SAL) affects performance-related regulation of ACh release, as higher levels of ACh release were required to maintain normal performance levels in these animals.

As pointed out in the Introduction, the effects of repeated AMPH exposure model essential neurobiological and

behavioral/cognitive aspects of schizophrenia. The present results indicate that repeated AMPH exposure causes a fundamental loss of cognitive task control. Such failure of cognitive control has been proposed to form a general basis for the diverse cognitive symptoms of schizophrenia (Braver *et al*, 1999). Therefore, the effects of repeated AMPH exposure on attentional performance and performance-associated ACh release appear to form a useful model for further investigations on the cholinergic mechanisms underlying the cognitive impairments of schizophrenia. Furthermore, as ongoing experiments indicate the sensitivity of this animal model in terms of detecting the pro-cognitive effects of drug treatments (Martinez *et al*, 2006), this model may serve as a tool for research on the role of cholinergic mechanisms mediating the beneficial cognitive effects of treatments, and also for the detection of such treatments.

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