

Augmented Prefrontal Acetylcholine Release during Challenged Attentional Performance

Rouba Kozak¹, John P. Bruno² and Martin Sarter¹

¹Department of Psychology, University of Michigan, Ann Arbor, MI, USA and ²Departments of Psychology and Neuroscience, Ohio State University, Columbus, OH, USA

Previous research has demonstrated that attentional performance depends on the integrity of the cortical cholinergic input system and that such performance is associated with increases in cortical acetylcholine (ACh) release. The present experiment tested the hypothesis that the attentional impairments produced by bilateral basal forebrain infusions of the NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid (APV) are associated with attenuation of performance-associated increases in ACh release. Rats were trained in a sustained attention task and equipped with three guide cannula for the bilateral infusion of the NMDA receptor antagonist APV (0, 3, 20 nmol) and for the insertion of a dialysis probe into the medial prefrontal cortex (mPFC). APV or vehicle was infused remotely following completion of the first of five blocks of trials. During the first block, attentional performance was associated with a 140% increase in ACh efflux. Infusions of APV decreased the animals' ability to detect signals and augmented the increases in ACh efflux observed prior to infusions. These data indicate a dissociation between levels of attentional performance and increases in mPFC ACh release. Augmentation of performance-associated increases in mPFC cholinergic transmission is hypothesized to mediate the increased demands on attentional 'effort' that are required to maintain performance under challenging conditions.

Keywords: acetylcholine, attention, prefrontal cortex, basal forebrain, microdialysis

Introduction

The detrimental effects of lesions of cortical cholinergic inputs on attentional performance and the demonstration of attentional performance-associated increases in cortical acetylcholine (ACh) release have supported the hypothesis that the cortical cholinergic input system represents a core component of the telencephalic circuitry which mediates attentional processes and capacities (Everitt and Robbins, 1997; McGaughy *et al.*, 2000; Hasselmo and McGaughy, 2004; Sarter *et al.*, 2005). Importantly, the increases in cortical ACh release that were observed in attention task-performing animals were not found in animals performing behavioral control procedures that matched or exceeded the demands on basic behavioral operations but were devoid of explicit demands on attention (Himmelheber *et al.*, 1997; Dalley *et al.*, 2001; Arnold *et al.*, 2002).

Although the present evidence consistently indicates that, following the removal of confounding effects of novelty and stress-related manipulations, demands on attentional processes or capacities are necessary for the demonstration of robust increases in cortical acetylcholine (ACh) efflux (Pepeu and Giovannini, 2004), attempts to systematically vary cortical ACh efflux as a function of demands on attention have largely failed to specify the precise behavioral or cognitive operations that cause

increases in cortical ACh release in task-performing animals. For example, variations of signal duration in the five-choice serial reaction time task produced the expected effects on performance but were not correlated with systematic changes in medial prefrontal ACh efflux (Passetti *et al.*, 2000). The data from two other experiments likewise indicated that limited variations in levels of attentional performance were not associated with robust changes in performance-associated increases in ACh efflux (Himmelheber *et al.*, 2001; McGaughy *et al.*, 2002).

Therefore, the present experiment was designed to generate impairments in attentional performance and to test the hypothesis that performance-associated increases in medial prefrontal cortex (mPFC) ACh release are attenuated during impaired performance. We previously demonstrated that bilateral infusions of an NMDA receptor antagonist into the basal forebrain (BF) results in impairments in the detection of signals in an operant sustained attention task (Turchi and Sarter, 2001). Studies measuring cortical ACh release in non-performing animals demonstrated that such infusions decrease basal cortical ACh efflux (Rasmusson *et al.*, 1996; Giovannini *et al.*, 1997). In rats which were extensively handled and habituated to the experimental testing conditions, perfusions of the ionotropic glutamate receptor antagonist kynurenatate into the basal forebrain did not affect the basal release of ACh in the cortex but attenuated increases in ACh release that were produced by the presentation of a complex stimulus consisting of lights-out and the presentation of palatable food (Fadel *et al.*, 2001). Based on this evidence indicating, collectively, that basal forebrain ionotropic glutamate receptor blockade impairs attentional performance and decreases basal cortical ACh efflux and/or attenuates increases in ACh efflux, attentional performance-associated increases in cortical ACh efflux were expected to be attenuated by basal forebrain infusions of an NMDA receptor antagonist. In the present experiment, an NMDA receptor antagonist was infused bilaterally into the BF of task-performing rats, using a remote infusion method. Performance-associated mPFC ACh efflux was measured in these animals using microdialysis. Contrary to the original hypothesis, results indicate robust increases in ACh efflux as a result of BF NMDA receptor blockade and during the manifestation of impairments in performance. These data form the basis for a new hypothesis about the contributions of mPFC cholinergic inputs to the mediation of attentional performance.

Materials and Methods

Animals and Animal Housing

The subjects were male Fischer-344/Brown-Norway F1 hybrid rats (Harlan Sprague-Dawley, Indianapolis, IN) aged 2 months at beginning of the behavioral training and between 9 and 12 months during the

microdialysis sessions. Animals were individually housed in a temperature (23°C) and humidity-controlled (45%) environment and on a 12h light/dark cycle (lights on at 6.30 a.m.). Animals were extensively handled prior to the beginning of training, and were water-deprived by restricting access to water to an 8 min period after each daily behavioral session. Water was also provided as a reward during task performance (see below). Food was available *ad libitum* (Rodent Chow, Harlan Teklad, Madison, WI). Animal care and experimentation were performed in accordance with protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee (ILACUC) and were in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals*.

Apparatus and Behavioral Training Procedures

Behavioral training and testing was conducted using a set of eight operant chambers (Med-Associates, St Albans, VT), located inside larger sound-attenuating chambers. Each operant chamber was equipped with an intelligence panel consisting of three panel lights (2.8 W), two retractable levers and a water dispenser (40–45 μ l water per delivery). A house light (2.8 W) was located on the rear wall. Prior to and during sessions involving microdialysis (see below), animals were trained/ tested using four modified operant chambers. These boxes featured a taller recessed water delivery area (9.0 \times 5.0 cm, height \times width) to allow cannulated animals to drink and an opening in the ceiling of the operant chamber and sound attenuating chamber to allow for the placement of liquid swivels, syringes, and microdialysis and infusion tubing outside the chambers. This arrangement permitted the collection of dialysates outside the chambers and the remote infusion of drugs, while not interfering directly with the animals' continuing performance. Signal presentation, lever operation, reinforcement delivery and data collection were controlled by a Pentium PC and Med-PC for Windows software (V 4.1.3; Med-Associates).

Animals were initially shaped to lever-press in accordance with a modified FR-1 schedule for water reinforcement. Following at least three consecutive sessions/days of >100 reinforced lever presses, animals entered the first stage of sustained attention task training. The task and evidence in support of the validity of performance measures in terms of indicating sustained attention performance were described previously (McGaughy and Sarter, 1995).

In the first stage of training, animals were required to discriminate between signals (1 s illumination of the central panel light) and non-signal (no illumination) events. Each response period was cued by extension of the levers into the chamber 2 s after a signal or non-signal event. On signal trials, a response on the left lever was reinforced and termed a 'hit' while a response on the right lever was not reinforced and termed a 'miss'. Half of the animals were trained in accordance with the reversed rules. On non-signal trials, a response on the right lever was reinforced and termed a 'correct rejection' while a response on the left lever was termed a 'false alarm' and not reinforced. If no response occurred within 4 s, the levers were retracted and an omission was recorded. Signal and non signal events were presented in pseudo-random order for a total of 81 trials each per session. The inter-trial interval (ITI) was 12 ± 3 s. During this stage of training, incorrect responses were followed by up to three correction trials in which the previous trial (signal or non signal) was repeated. In the event of an incorrect response on the third correction trial, a forced trial was initiated in which only the correct lever was extended for 90 s or until the animal made a response. When the forced trial was a signal trial, the central panel light was also illuminated while the left lever was extended. The house-light was not illuminated during this training step.

Following at least five consecutive days of stable performance, defined as >70% hits to longest signals and >70% correct rejections, multiple signal durations (500, 50, 25 ms) were introduced. Trial type and signal duration continued to be pseudo-randomly determined for each trial. Session length was set at 40 min to correspond with the timing of subsequent dialysate collections (five collections over 8 min each). The pseudo-random selection of trial type (signal versus non-signal) and signal duration was designed to ensure that approximately half of the trials per 8 min block were signal trials, and that equal numbers of 500, 50 and 25 ms signals were presented during each block. At this point, correction trials and forced trials were discontinued, and the event rate

was increased by reducing the ITI to 9 ± 3 s. Following at least 7 days of stable performance (at least 70% hits to 500 ms signals, at least 70% correct rejections and <50% omissions to 25 ms signals), animals began training in the final version of the task (see Fig. 1A).

Beginning with this final stage, house lights were illuminated throughout the session. This important final modification requires the animals to constrain their behavior and, presumably, to maintain persistent attention to the intelligence panel for signal detection, and thus resulted in several weeks of additional training until the animals reached criterion performance. Final criterion performance was defined as >65% hits to 500 ms signals, >65% correct rejections and <20% omissions for seven consecutive sessions.

After attaining criterion performance, additional daily training sessions were conducted in the modified operant chambers (above). Procedures designed to foster habituation to the microdialysis and infusion procedures were initiated at this point. Rats were placed in these chambers 212 min prior to task onset (Fig. 1B). During subsequent microdialysis sessions, this time period corresponded with 3 h of discard period after the probe insertion, and an additional 32 min period that was used prior to task onset to collect four samples for the determination of basal ACh efflux (B1–B4 in Fig. 3). Furthermore, after the completion of the task, animals remained in the chambers for an additional 1 h for the collection of six post-task dialysates. House lights remained turned on during the entire pre-task, task, and post-task period. After attaining stable performance (as defined above) in these chambers for at least 3 days, animals underwent cannulation surgery.

Behavioral Measures

Measures of performance included hits, misses, correct rejections, and false alarms. The relative number of hits (hits/hits + misses) was calculated for each signal length, and the relative number of correct rejections (correct rejections/correct rejections + false alarms) was also calculated. Finally, errors of omission were recorded. These measures were calculated for the entire 40 min task as well as for each of five task blocks (8 min each).

Surgical Methods

Surgery was performed under aseptic conditions. Initial anesthesia was induced with 4–5% isoflurane and by placing the animal in an anesthetic chamber (Anesco/Surgivet, Waukesha, WI). Gas was carried via oxygen at a flow rate of 0.6 ml/min. Animals were also given preoperative injection of an antibiotic (Amoxicillin, 100 mg/kg; s.c.). The animals' heads were shaved using electric clippers and cleaned with 70% ethanol and Nolvason, a topical antiseptic. Ophthalmic ointment was used 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.

Guide cannula (26 gauge, o.d. 0.46 mm; Plastics One, Roanoke, VA) were implanted into both hemispheres (hs) according to the following coordinates (Paxinos and Watson, 1988), relative to Bregma: AP: -1 mm; ML: ± 3.5 mm, at an angle of 4° toward the midline; DV: -6.4 mm beneath the skull surface. These coordinates placed the tips of the guide cannula 1 mm above the area of substantia innominata (SI) of the BF. Microdialysis guide cannulas were implanted above the medial prefrontal cortex (mPFC) at the following coordinates: AP: 4.2 mm; 20° rostral angle, ML: 0.6 mm, DV: 0.6 mm below dura. In order to prevent clogging of the cannula shafts, dummy cannula were inserted into infusion guide cannula, and a stainless steel stylets into the microdialysis guide cannula. After surgery, rats were returned to their home cages and allowed to recover for 7 days with free access to food and water. Following the post-operative recovery period, the water deprivation schedule was resumed and animals were returned to behavioral training until they regained criterion performance. At this stage, dummy cannula and the stylet were removed and polyethylene tubing was attached during additional training sessions in order to habituate the animals to the final microdialysis conditions and the infusion procedures.

Drug Infusions and Dialysate Collections

Once animals regained stable performance (see above for criteria), test sessions that included intracranial drug infusions and dialysate collections were conducted. The present design required three dialysis

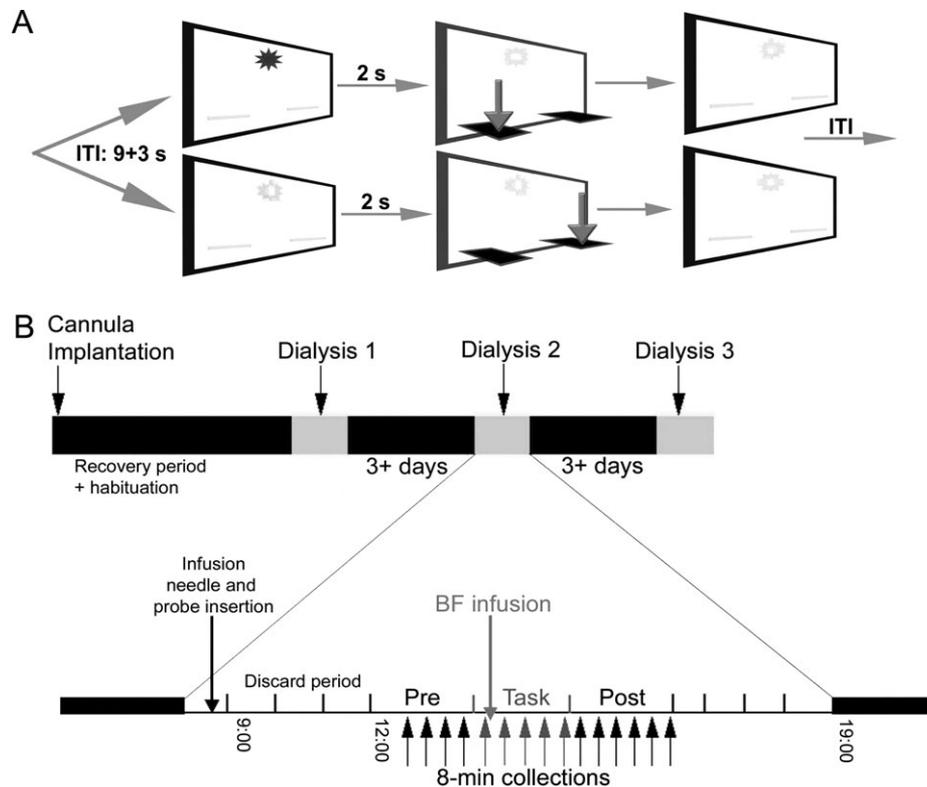


Figure 1. Illustration of the main components of the sustained attention task (A), summary of the main experimental events and illustration of the detailed sequence of events during an individual dialysis session (B). (A) Following a variable ITI, a signal (illumination of a panel light for 500–25 ms) was presented (top sequence) or not (non-signal; lower row). Both levers were extended 2 s later and animals were required to press one lever to report a hit and the other one to report a correct rejection to receive reward (see arrows exemplifying one set of rules). Incorrect responses (misses, false alarms) and omission were not rewarded. Levers were withdrawn following a lever press or after 4 s. (B) As detailed in Materials and Methods, animals were implanted with three guides and, during a subsequent recovery period and daily behavioral training sessions, habituated to performing the task while dummy cannula were inserted and connected to syringes and pumps located outside the sound-attenuating chambers. Probes were inserted into the prefrontal cortex and perfused, infusion needles were inserted into the BF, and saline, 3 or 30 nmol of APV, respectively, was infused immediately after the collection of the first performance-related dialysate (T1; see insert). During an individual dialysis session, the probe and the infusion needles were inserted early in the morning, the animal was placed into the operant chamber, and probes and needles were connected to syringes and pump. During the next 3 h, the probe was perfused and dialysates were discarded. The four collections prior to task onset were used to determine the stability of ACh efflux and basal ACh efflux. Five 8 min collections were taken during task performance. BF infusions were carried out immediately after completion of T1. An additional six collections were taken after completion of the task.

sessions. If individual dialysis sessions did not generate detectable levels of ACh efflux because of probe failure during dialysis or because probe tubing had been severed, a maximum of two additional sessions was conducted in order to generate a complete data set from an individual animal and to maintain a within-subject design. Our previous experiments supported the validity of the cortical ACh efflux data generated from multiple sessions and by repeated insertions of probes, both of which were separated by several days (Moore *et al.*, 1995, 1999). Each dialysis session started by the removal of the dummy cannula and the insertion of the internal infusion needles (33-gauge, i.d.: 0.10 mm; Plastics One, Roanoke, VA) into the guide cannula placed into the BF. Infusion cannula were cut to project 1 mm beyond the tip of the guide cannula into the substantia innominata (SI). Cannula were connected to the tubes (Model CMA/11, i.d.: 0.12 mm; CMA, Solna, Sweden) that contained 0.5 μ l of drug or vehicle. Using a Hamilton 10 μ l microsyringe, drug or vehicle was pulled back from the tip of the infusion needles, producing a dead volume of \sim 0.5 μ l.

The stylet was also taken out and a removable concentric probe with a 3.0 mm membrane tip (Model: MAB4; membrane o.d.: 0.24 mm; Scientific Products and Equipment, Stockholm, Sweden) was inserted through the guide cannula into the PFC. An estimate of probe efficiency was obtained *in vitro*, to ensure adequate recovery before probe insertion, by placing the probe in a standard ACh solution (1.0 pmol) and taking one 8 min collection. Animals were perfused at a rate of 2.0 μ l/min with artificial cerebrospinal fluid (aCSF), 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.

Rats were placed into the operant chambers for 212 min prior to task onset (Fig. 1); probes were perfused for 180 min to allow ACh efflux to stabilize. Collections of samples began 32 min prior to the task onset. Dialysates were collected every 8 min. The last four collections prior to task onset (B1–B4) were used to calculate basal ACh efflux. Following the onset of the task, the timing of dialysates collections was adjusted to correct for the dead volume of the probe and outlet tubing. After the collection of the first performance-associated 8 min dialysate, drug or vehicle was infused (below). Four additional samples were collected following drug infusion and while the animal performed the task. Six additional dialysates were collected following the completion of the task and while animals remained in operant chambers. Thereafter, infusion needles and the microdialysis probes were removed, dummies and stylets inserted, and the rats were returned to their cages.

Drugs and Infusion Parameters

Rats received bilateral infusions of either vehicle (saline; 0.9%) or the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV; RBI, Natick, MA; 3 or 20 nmol in 0.5 μ l/hemisphere; Turchi and Sarter, 2001). The order of infusions was counterbalanced, with at least 2 days separating two successive sessions, or as many sessions as was required for rats to regain stable baseline performance.

Following the collection of the first performance-associated sample (T1; see Fig. 1), APV or vehicle was injected into the BF. Drug stock solutions were diluted with HPLC grade water to a concentration of 10 mM. This solution was aliquoted into dark vials and kept frozen at -70°C . Prior to use, on the day of infusions, the frozen aliquot was thawed and stock solution was diluted with saline to the desired concentration. All

solutions were at room temperature at the time of infusion. A 10 μ l Hamilton microsyringe was attached to the polyethylene tubing. An infusion pump (Model CMA/100, Solna, Sweden) was used to make the simultaneous bilateral infusions of 1 μ l/hemisphere over a 2 min period at rate of 0.5 μ l per min (the total volume injected consisted of 0.5 μ l dead volume plus 0.5 μ l drug or vehicle). Infusions of 0.5 μ l have been calculated to diffuse into an area of \sim 0.5 mm diameter (Routtenberg, 1972). Thus, similar to our previous studies (Turchi and Sarter, 2001), infusions of APV or saline were assumed to diffuse maximally into the ventromedial globus pallidus and the dorsal aspects of the horizontal limb of the diagonal band (see Fig. 2 for additional evidence based on infusions of FluoroGold into the BF).

Determination of ACh Concentrations

Dialysate samples were frozen at -80°C until analyzed by high-performance liquid chromatography with electrochemical detection (ESA, Chelmsford, MA), using a mobile phase containing 100 mM sodium

phosphate, 0.5 mM tetramethylammonium chloride and 2 mM 1-octanesulfonic acid. ACh was separated from choline on a 250 mm analytical column 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. 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/13 μ l injection.

Histological Verification of Probe and Cannula Placements

Within 1 week following the last microdialysis session, animals were given an overdose of sodium pentobarbital and perfused with ice-cold saline (0.9%) containing 0.2% heparin, 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) surrounding the probe and cannula sites were mounted, stained with cresyl violet, and examined for cannula and probe placements.

Statistical Analyses

Performance Data Analyses

Complete sets of data were available from six rats for final analyses. Analyses of the hits and correct rejections were carried out using angularly transformed values (Zar, 1974). Performance data from the day prior to each dialysis session were averaged and used for baseline comparisons. To assess differences between baseline performance and the effects of saline, repeated-measures analysis of variance (ANOVA) was conducted to determine the effects of saline (two levels), blocks (five levels) and signal duration (three levels) on hits, and the effects of saline and block on correct rejections and omissions. Repeated-measures ANOVA was also used to assess the effects of APV (three levels: saline, 3 nmol, 20 nmol), block (five levels) and signal duration (three levels) on hits, correct rejections and omissions (the analysis of the two latter measures did not include the factor 'signal duration'). Significant interactions indicated by these analyses were followed up by multiple one-way ANOVAs as a first step toward locating the source of significant *F*-statistics. Dependent *t*-test and simple contrasts for within-subjects factors were used to determine the specific source for significant interactions.

Analysis of ACh Concentrations

ACh efflux measures were not corrected for probe recovery (10–15%). Basal ACh efflux data from sessions involving the subsequent infusion of saline, 3 or 20 nmol APV were compared using repeated measures ANOVA on the effects of drug (three levels) and collection (four levels). Demonstrating that basal efflux prior to drug infusions was identical across sessions permits the expression of drug infusion-induced changes in performance-associated ACh efflux as a percent change from basal ACh efflux. All subsequent statistical analyses were performed on data expressed as a percent change from the mean basal ACh efflux. To determine drug infusion-induced changes in performance-associated ACh efflux, data were analyzed using repeated-measures ANOVA involving the factors drug (three levels) and block (five levels). Significant interactions were followed up by multiple one-way ANOVAs and multiple comparisons using dependent *t*-tests.

General Statistical Methods

The reported statistical results reflect Huyn-Feldt-corrected degrees of freedom (Vasey and Thayer, 1987). Exact *P* values were reported (Greenwald *et al.*, 1996). Statistical analyses were performed using SPSS/PC (V+11.5; SPSS Inc., Chicago, IL).

Results

Dialysis and Infusion Cannula Placements

Figure 2 illustrates and exemplifies the placement of dialysis probes in the mPFC and of infusion cannula in the BF (see

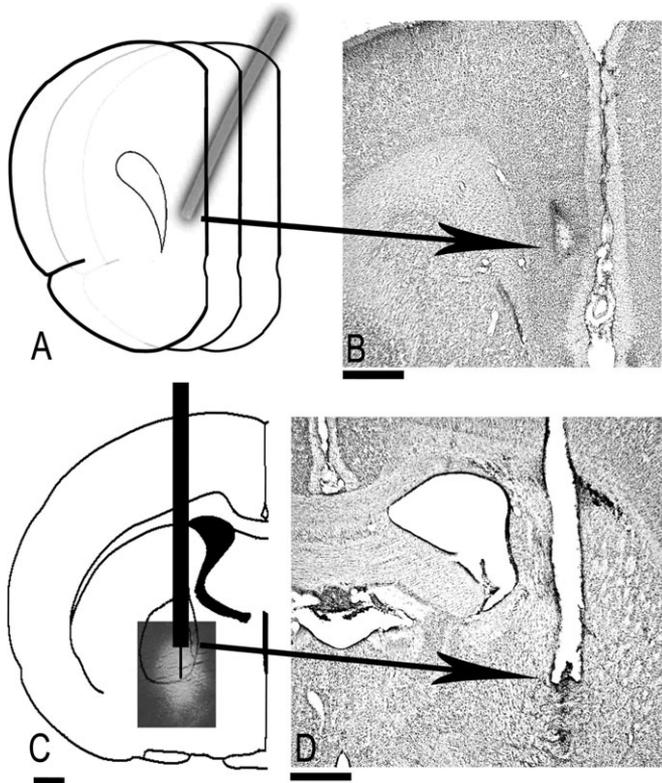


Figure 2. Illustration and examples of the placement of probes into the medial prefrontal cortex and guide cannula and infusion needles into the BF. (A) A schematic illustration of the placement of probes (3 mm membranes) into the prefrontal cortex at a 20° anterior-posterior angle. As coronal sections were cut at no angle in order to visualize BF cannula placements (C, D), prefrontal sections representing the dorsal-ventral placements of probes are unavailable. (B) The location of the posterior-ventral placement of the tip of the probe in the prelimbic cortex. Based on the placement of probes and prior data examining the approximate region perfused by such probes (Neigh-McCandless *et al.*, 2002), ACh was collected from anterior cingulate and prelimbic regions. (D) An example showing the placement of guide cannula for the insertion of infusion needles into the BF. The tip of the guide is readily visible (arrow), and the tract produced by the infusion needle is also apparent. In (C), the diameters of the schematically drawn guide cannula and infusion needle are drawn to approximate scale. Furthermore, a microphotograph of a section showing the spread of FluoroGold (0.5 μ l; 0.1%; FluoroChrome, Englewood, CO) infused into the BF was superimposed and adjusted to scale. Although the different physico-chemical properties of FluoroGold limit the generalization of these data, the spread of the tracer suggested that the infusions affected primarily the ventro-medial globus pallidus that contain the cholinergic neurons of the ventral nucleus basalis of Meynert and the substantia innominata. Furthermore, the spread of the tracer conformed with other evidence about the spread of intraparenchymal drug infusions (Routtenberg, 1972; Pecina and Berridge, 2000).

legend for details). ACh was collected from ventral portions of the anterior cingulate cortex and from prelimbic regions. The tips of infusion needles were placed in the ventral globus pallidus, dorsal to the substantia innominata (SI) portion of the BF. The spread of Fluorogold infusions (see Fig. 2C and legend for details) suggested that intra-BF infusions spread along the ventral medial wall of the globus pallidus and thus included the nucleus basalis of Meynert and the substantia innominata.

Performance-associated ACh Efflux

Basal ACh Efflux

The average ACh concentrations in the last four 8 min dialysate collections taken prior to task onset were used to determine basal ACh efflux. Basal ACh efflux values obtained from this period prior to the three test sessions conducted per animal (saline, APV 3 nmol, APV 20 nmol) were compared and found not to differ statistically [$F(2,10) = 0.53$; $P = 0.61$]. Averaged across animals and test sessions, basal ACh efflux was 5.35 ± 1.31 fmol/13 μ l. Figure 3 (top) depicts ACh efflux, expressed as

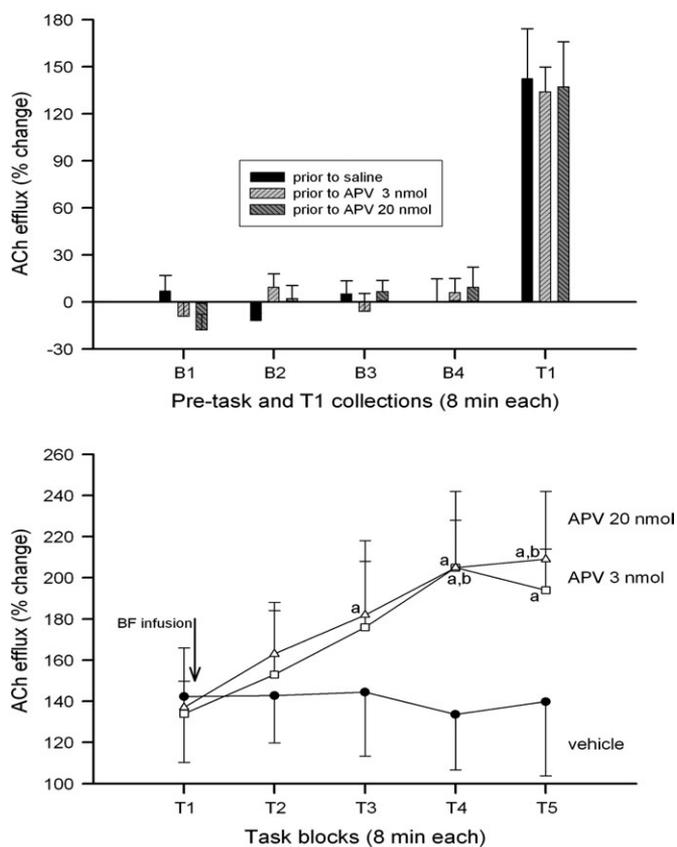


Figure 3. Basal and attentional performance-associated changes in ACh efflux (mean \pm SEM). The top graph illustrates ACh release during the four collections intervals prior to task onset (B1–B4), separated by dialysis session type (or infusion condition; saline, APV 3, 20 nmol). Additionally, the average increase in ACh release during the first 8 min task block, and prior to the infusions of saline or APV (T1), is depicted. ACh efflux was significantly increased during T1 when compared with baseline, and this increase did not differ between animals that received saline or APV immediately after the collection of T1 dialysates. As shown in the lower graph, the performance-associated increase in ACh efflux remained relatively stable following the infusion of saline into the BF. In contrast, further increases in ACh efflux were observed following infusions of 3 or 20 nmol of APV, and while the animals' performance was impaired as a result of APV infusions [a, significantly different from T1; b, significantly different from T2 ($P < 0.05$), as indicated by dependent t -tests].

percent change from basal ACh efflux, during the four collections prior to task onset (B1–B4) and during the first block of trials and prior to infusions of saline or APV into the BF (T1).

Performance-associated ACh Efflux prior to BF Infusions (T1)

The first collection of ACh in attention task-performing animals (T1 in Fig. 3, both graphs) occurred prior to the remote infusion of saline or APV into the BF. ACh concentrations in T1 dialysates were analyzed separately to confirm the absence of session-related differences in performance-associated ACh efflux prior to the BF infusion of saline or APV. During T1, ACh efflux did not differ between drug conditions [$F(2,10) = 0.02$; $P = 0.98$]. During T1, ACh efflux was increased by $138.20 \pm 14.24\%$ of baseline (Fig. 3, top and bottom graphs). Compared with basal ACh efflux, T1 ACh efflux was significantly increased [$t(5) = 10.16$; $P < 0.001$].

ACh Efflux across Task Blocks and BF Infusion Conditions

ACh concentrations in the five collections that were taken at the end of each of the five 8 min blocks of attentional task performance (T1–T5) were analyzed for the effect of task blocks and BF infusion condition (saline, APV 3 or 20 nmol; Fig. 3, bottom graph). This analysis yielded a main effect of task block and a significant interaction between the effects of task block and drug. The main effect of task block on ACh efflux [$F(4,20) = 8.25$; $P < 0.001$] reflected a task block-related increase in ACh efflux. However, as illustrated in the lower graph of Figure 3, this effect was based exclusively on task block-related increases in ACh efflux observed following the remote BF infusions of APV (3 or 20 nmol), but not following infusions of saline [block \times drug: $F(7.45, 37.2) = 2.35$; $P = 0.04$]. *Post hoc* one-way ANOVAs on the effects of task block on ACh efflux confirmed the absence of changes in ACh efflux across blocks following infusions of saline [$F(3.15, 15.73) = 0.14$; $P = 0.94$]. Performance-associated ACh efflux following saline infusions mirrored previous results from experiments assessing ACh efflux in (untreated) attentional task-performing rats (Arnold *et al.*, 2002). Furthermore, *post hoc* one-way ANOVAs confirmed that significant increases in ACh efflux occurred following the infusion of the lower [$F(3.43, 17.15) = 4.16$; $P = 0.019$] as well as the higher [$F(4,20) = 9.33$; $P < 0.001$] concentration of APV (Fig. 3, lower graph). Multiple comparisons indicated that following infusions of 3 nmol APV, ACh efflux was higher during the last two collections (T4, T5) when compared with T1 [$t(5) = 5.32$; $P = 0.003$; $t(5) = 7.50$; $P = 0.001$], and T4 efflux was also higher than T2 efflux [$t(5) = 2.72$; $P = 0.042$]. Infusions of 20 nmol APV caused T3, T4, and T5 ACh efflux to be higher than T1 efflux [$t(5) = 3.60$; $P = 0.015$; $t(5) = 4.49$; $P = 0.004$; $t(5) = 5.68$; $P = 0.002$, respectively] and T5 ACh efflux was also higher than T2 efflux [$t(5) = 3.45$; $P = 0.018$]. Thus, performance-associated ACh efflux remained stable across the entire task session following infusions of saline into the BF. In contrast, following infusion of the NMDA receptor antagonist APV, ACh efflux increased significantly during the last two (3 nmol) and three (20 nmol) task blocks, respectively.

Post-task ACh Efflux

Following completion of the performance component of a test session, animals remained in the operant chambers for the collection of six additional 8 min dialysates (see Fig. 1). Post-task ACh concentrations declined across post-task collections [main effect of block: $F(2.91, 14.55) = 5.31$; $P = 0.012$], but this decline

did not differ between sessions assessing the effects of saline, 3 or 20 nmol of APV [block \times drug: $F(7.85,39.25) = 2.01$; $P = 0.07$]. ACh efflux during the final post-task collection interval was $36.60 \pm 16.84\%$ of baseline and was no longer significantly different from basal ACh efflux [$t(5) = 2.10$; $P = 0.09$].

Attentional Performance following Infusions of Saline or APV into the BF

Baseline Performance and Effects of BF Saline Infusions

The relative number of hits was signal duration-dependent [$F(1.91,9.58) = 28.11$; $P < 0.001$], reflecting the decrease in hit rates with shorter signal duration that has been routinely observed in animals performing this task (Fig. 4). Furthermore, a significant interaction between the effects of task block and signal duration [$F(5.56,27.78) = 3.18$; $P = 0.019$] was indicative of a steeper decrease in hits to longest signals across trial blocks when compared to shortest signals. This interaction was possibly confounded by a 'floor' effect with respect to the hit rate to 25 ms signals (not shown). Animals generated $62.76 \pm 2.28\%$ correct rejections and omitted 7.76 ± 1.71 trials per session.

Compared with baseline performance, infusions of saline into the BF did not affect the animals' hit rate [$F(1,5) = 0.89$; $P = 0.39$; Fig. 4]. Furthermore, saline infusions did not affect the animals' ability to respond correctly in non-signal trials or the number of omissions (all P s > 0.1). The effects of APV infusions on attentional performance were compared with the effects of saline infusions into the BF.

Effects of APV Infusions

Infusions of APV into the BF impaired the animals' ability to detect signals, and this effect of APV depended on signal duration and task-block [$F(15.49,77.45) = 2.07$; $P = 0.02$]. Inspection of the hit rates to the three signal durations suggested that this interaction was due to a decrease in hits following APV infusions in trials presenting 500 ms signals (Fig. 5). Following infusions of saline, the relative number of hits to 500 ms did not change across trial blocks (see Fig. 5). In contrast, following the infusions of 3 nmol APV, the animals' hit rate decreased immediately after the infusion and during T2, and recovered during the remaining blocks. In contrast, following

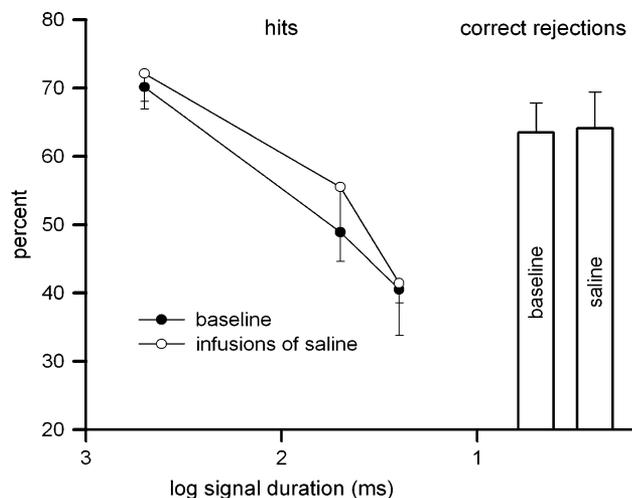


Figure 4. Performance at baseline and following infusions of saline into the BF. In signal trials, the number of correct responses (hits) varied with signal duration. Infusions of saline did not produce main effects on performance.

infusions of 20 nmol APV, the animals' hit rate remained at $\sim 40\%$ during the remaining blocks of trials. *Post hoc* multiple comparisons, based on the tests for simple contrasts of within-subjects data, indicated that following infusion of the higher dose of APV, the animals' hit rate during T4 was lower than prior to the infusion [$F(1,5) = 6.59$; $P = 0.050$]. Infusions of APV did not affect the animals' ability to respond correctly in non-signal trials [$F(2,10) = 0.18$; $P = 0.84$] and they did not affect the number of omissions [$F(2,10) = 10.47$; $P = 0.64$].

Discussion

Attentional performance-associated ACh efflux increased by $\sim 140\%$ during the first block of trials. Subsequent, remote infusions of saline into the BF did not alter performance-associated ACh efflux during the remaining four blocks of trials. Unexpectedly, the impairments in performance produced by APV infusions were associated with further, significant increases in mPFC ACh efflux. The augmenting effects of APV on ACh release in task-performing animals contrast with the attenuation of increases in ACh release, or even decreases in basal ACh release, which were observed in non-performing animals following BF NMDA receptor blockade (Rasmusson *et al.*, 1996; Giovannini *et al.*, 1997; Fadel *et al.*, 2001).

During the first block of task performance (T1), and following the infusion of vehicle into the BF, performance-associated mPFC ACh efflux remained stable at $\sim 140\%$ of baseline. Our previous experiment (Arnold *et al.*, 2002) reported higher extracellular levels of ACh in performing animals ($\sim 200\%$), presumably because of the addition of a cholinesterase inhibitor ($0.1 \mu\text{M}$ neostigmine) to the perfusion medium in the former,

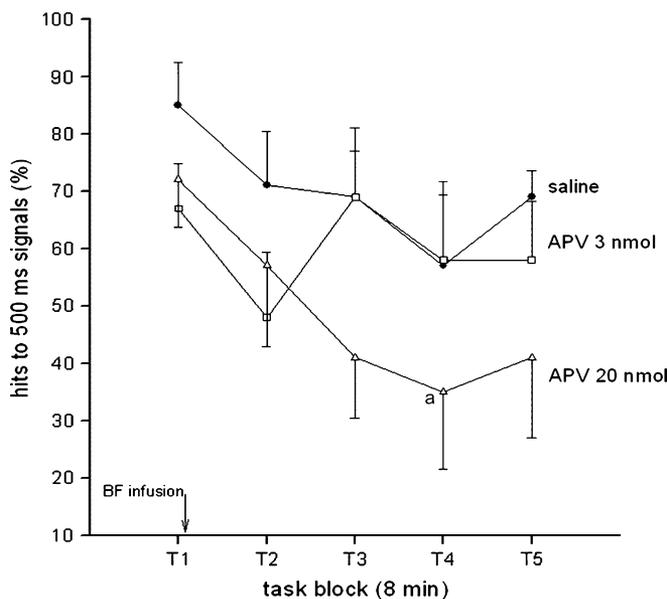


Figure 5. The effects of infusions of the NMDA receptor antagonist APV into the BF on the relative number of hits to 500 ms signals. This figure shows the relative number of hits to 500 ms signals, prior and following the perfusion of saline, 3 or 20 nmol of APV (see the arrow marking the infusion time), across blocks of trials. Following infusions of the lower dose of APV, decreases in hits to 500 ms signals did not reach statistical significance and appeared to have fully recovered two blocks following infusion. Hit rates to 500 ms signals remained depressed throughout the remainder of the session following infusions of the higher concentration of APV (a, significantly different from T1). Hits to 50 and 25 ms signals were not systematically affected by APV (not shown), thereby giving rise to a significant interaction between the effects of APV, block and signal duration.

but not present, experiment. Similar to the previous data, ACh efflux in saline-infused animals did not vary across task blocks.

BF infusions of APV resulted in a reduced hit rate to longest signals. Detrimental effects on the detection of hits typically manifest with respect to longest signals (McGaughy *et al.*, 1996; Turchi and Sarter, 2001), in part because 'floor' effects often limit the demonstration of decreases in hits to shorter signals. *Post hoc* multiple comparisons did not indicate a significant effect of the lower dose of APV (3 nmol). While Figure 5 suggests a transient decrease in hits during one task block immediately after the infusion of this dose, the higher dose (20 nmol) resulted in a more lasting impairment, yielding an ~30% decrease in hits to longest signals during post-infusion task blocks. Compared with previous data on the effects of BF APV infusions on attentional performance (Turchi and Sarter, 2001), the variability of the present effects on performance was higher, possibly in part as a result of the concurrent microdialysis procedure.

The present experiment was the first to measure performance-associated ACh efflux while drug was infused bilaterally in order to alter the excitability of the basal forebrain and to produce distinct performance effects. Furthermore, drug was infused remotely after the first block of trials while animals were performing, thereby allowing the analyses of data from the first block of trials, to ensure regular baseline performance and regular performance-associated increase in ACh release for each dialysis session and prior to BF infusions. This point deserves emphasis because, based on this challenging methodological approach, the unexpected increases in ACh efflux following APV infusions are known not to have been confounded by abnormal levels of performance-associated increases in ACh efflux prior to the infusions (see Fig. 3).

Following the infusion of both concentrations of APV, performance-associated ACh efflux increased further during the remaining four blocks of tasks (or 32 min), reaching ~200% of baseline during the last two blocks of trials. Importantly, post-task ACh release returned to basal (pre-task) levels irrespective of BF infusions, supporting the hypothesis that the increases in ACh efflux following APV were due to interactions between task performance and drug. The latter point is also supported by the fundamentally opposite effects of BF NMDA receptor blockade on ACh efflux in non-performing animals (Rasmusson *et al.*, 1996; Giovannini *et al.*, 1997; Fadel *et al.*, 2001).

Functions of Augmented Increases in ACh Efflux

The available literature provides little insight into the nature of the behavioral or cognitive variables that may have been responsible for the augmentation of performance-associated ACh efflux observed during APV-induced impairments in performance. However, a transient augmentation of performance-associated ACh efflux was previously observed following the brief presentation of a distractor and while animals' performance recovered from the impairment in performance (Himmelheber *et al.*, 2000). This finding and the present data may be interpreted in support of the hypothesis that 'attentional effort', rather than levels of performance, predicts levels of increases in cortical ACh efflux.

Attentional 'effort' refers to the cognitive operations required to maintain performance under challenging and detrimental conditions. These operations include top-down mechanisms designed to optimize stimulus detection, discrimination and processing, to assign greater cognitive resources to the pro-

cessing of task-related information and to the preparation and execution of responses. Such executive functions are generally attributed to prefrontal networks, including the prefrontal modulation of cholinergic transmission elsewhere in the cortex (Sarter *et al.*, 2005). Conversely, cholinergically-enhanced stimulus processing elsewhere in the cortex reduces the demands on prefrontal participation (Furey *et al.*, 2000).

The effects of APV infusions on performance were selective for trial type (signal trials) and not indicative of a global disruption of performance, as non-signal trial performance and omissions remained unaffected. Thus, following APV infusions, the animals did not cease to perform but remained on task. This view is also supported by the performance recovery seen following infusions of the smaller concentration of APV (Fig. 5). If animals had stopped performing following APV infusions, a return of ACh efflux values to basal pre-task values would have been expected, as indicated by the finding that such a return occurred during the post-task period. Thus, the animals remained 'engaged' and continued performing despite the detrimental effects of APV. Therefore, augmented increases in ACh efflux can be interpreted as indicating that increased attentional 'effort' contributed to the animals' ability to sustain performance following APV infusions, and that such increases in 'attentional effort', rather than levels of performance, predict levels of performance-associated ACh efflux in the mPFC. Increases in 'attentional effort' involve motivational processes and, as discussed further below, neuronal circuitries which mediate the 'motivational recruitment' of the basal forebrain cholinergic system have been conceptualized.

Limitations of This Hypothesis

The present data do not indicate that different levels of performance recovery following APV infusions were associated with different levels of ACh efflux. Rather, efflux levels increased in parallel in animals following infusion of either concentration of APV. The variability of the performance data and the resulting, limited findings from *post hoc* multiple comparisons, which presumably were due in part to the complex experimental conditions and procedures, further limited the possibility of establishing direct performance-release relationships. The temporal resolution of ACh efflux data obtained by using microdialysis also contributes to the limited power of such experiments. However, the significance of the present finding would not be altered even if the behavioral effects of APV had remained insignificant. The finding that BF NMDA receptor blockade resulted in augmented increases in ACh efflux in task-performing animals while decreasing or attenuating ACh efflux in non-performing animals (Rasmusson *et al.*, 1996; Giovannini *et al.*, 1997; Fadel *et al.*, 2001) allows, at the very least, the conclusion that attentional performance was responsible for the 'paradoxical' effects of APV on ACh efflux. However, the present data do not indicate a relationship between levels of residual performance and increases in ACh efflux. It may be speculated that augmented increases in ACh efflux in principle do not correlate with performance levels, and the fact that animals following the infusion of either concentration of APV maintained relatively stable levels of performance may represent a sufficient predictor of the (similar) increases in ACh efflux. Ongoing experiments test the hypothesis that loss of prefrontal cholinergic inputs prohibits animals from maintaining such residual levels of performance following detrimental manipulations. Finally, it should be stressed that these considerations

and speculations apply specifically to the function of augmented increases of ACh efflux seen as a response of performance challenges, and they do not concern the conclusion from several experiments (see Introduction) which established that attentional performance, but not the performance of control tasks, depends on increases in ACh efflux.

Neuronal Circuitry Mediating Effort-associated Augmentation in ACh Efflux

Cholinergic inputs to prefrontal regions have been conceptualized to mediate the 'recruitment' of top-down mechanisms in situations characterized by increased demands on attentional processes and capacities (Sarter *et al.*, 2005). The results of neurophysiological experiments correspond with this view as they indicated that the cholinergic contributions to mPFC neuronal activity in task-performing animals manifest in response to challenging conditions (Gill *et al.*, 2000). Thus, the present data are hypothesized to reflect the cholinergic mediation of the recruitment of executive processes designed to maintain task performance under challenging conditions. This hypothesis also corresponds with the observation by Passetti *et al.* (2000) that the number of completed trials is a more effective predictor of levels of performance-associated ACh efflux than indicators of response accuracy.

The hypothesis that the present increases in mPFC ACh efflux reflect the increased demands on attentional 'effort' implies that the mechanisms that activate prefrontal cholinergic inputs under these conditions attenuated and in fact reversed the effects of BF NMDA receptor blockade. As already mentioned, in non-performing animals, BF NMDA receptor blockade attenuates increases in ACh efflux and was also reported to decrease basal ACh efflux (Rasmusson *et al.*, 1996; Giovannini *et al.*, 1997; Fadel *et al.*, 2001). In the present experiment, performance-associated increases in ACh efflux were not attenuated by APV infusions, but increased further when compared with pre-infusion levels. Increased activity of prefrontal (glutamatergic) projections to the basal forebrain (Zaborszky *et al.*, 1997) may have contributed to such a reversal of the effects of APV in performing animals, although the exact structure of this circuit remains unclear (Sarter and Bruno, 2002). Local intra-prefrontal mechanisms may also be capable in enhancing cholinergic synaptic transmission (Nelson *et al.*, 2005). Furthermore, non-glutamatergic mechanisms such as, for example, inhibition of adenosinergic transmission, may have contributed to the activation of the basal forebrain cholinergic system in performing animals and in response to APV-induced impairments in performance (e.g. Thakkar *et al.*, 2003). Finally, it also possible that residual performance under challenging conditions was associated with enhanced motivational processes which, mediated via increased activity in mesolimbic dopaminergic systems (Herberg *et al.*, 1976), might have contributed to cholinergic activation (Moore *et al.*, 1999; Neigh *et al.*, 2004). The determination of the neuronal mechanisms which, in performing animals, are capable of reversing the effects of NMDA receptor blockade on cortical ACh efflux will provide substantial insight in the mechanisms used by prefrontal regions to cope with increased demands on attentional performance.

Conclusions

The present evidence confirms that attentional performance is associated with robust (~140% over baseline) increases in

medial prefrontal ACh efflux. However, in contrast to non-performing animals exhibiting attenuating of ACh efflux following BF NMDA receptor blockade, ACh efflux in performing animals further increased (by an additional ~60%). Thus, the augmented increases in ACh efflux are a function of the animals' attentional performance. As animals' performance was impaired but not fundamentally disrupted, augmentation of performance-associated increases in ACh efflux is hypothesized to mediate the increased demands on attentional effort that was required for the animals to maintain (residual) performance. In contrast, termination of performance was associated with a return of ACh efflux levels to pre-task baseline values. It will be important that future experiments demonstrate that variations in the demands on effort are associated with different levels of prefrontal ACh efflux.

Notes

This research was supported by PHS Grants NS37026, MH63114 and KO2 MH01072. We are grateful to Vicente Martinez (University of Michigan) for his assistance concerning statistical analyses.

Address correspondence to Martin Sarter, University of Michigan, Dept. of Psychology, 525 East University Ave, Ann Arbor, MI 48109-1109, USA. email: msarter@umich.edu.

References

- Arnold HM, Burk JA, Hodgson EM, Sarter M, Bruno JP (2002) Differential cortical acetylcholine release in rats performing a sustained attention task versus behavioral control tasks that do not explicitly tax attention. *Neuroscience* 114:451-460.
- Dalley JW, McGaughy J, O'Connell MT, Cardinal RN, Levita L, Robbins TW (2001) Distinct changes in cortical acetylcholine and noradrenaline efflux during contingent and noncontingent performance of a visual attentional task. *J Neurosci* 21:4908-4914.
- Everitt BJ, Robbins TW (1997) Central cholinergic systems and cognition. *Annu Rev Psychol* 48:649-684.
- Fadel J, Sarter M, Bruno JP (2001) Basal forebrain glutamatergic modulation of cortical acetylcholine release. *Synapse* 39: 201-212.
- Furey ML, Pietrini P, Haxby JV (2000) Cholinergic enhancement and increased selectivity of perceptual processing during working memory. *Science* 290:2315-2319.
- Gill TM, Sarter M, Givens B (2000) Sustained visual attention performance-associated prefrontal neuronal activity: evidence for cholinergic modulation. *J Neurosci* 20:4745-4757.
- Giovannini MG, Giovannelli L, Bianchi L, Kalfin R, Pepeu G (1997) Glutamatergic modulation of cortical acetylcholine release in the rat: a combined *in vivo* microdialysis, retrograde tracing and immunohistochemical study. *Eur J Neurosci* 9:1678-1689.
- Greenwald A, Gonzalez R, Harris R, Guthrie D (1996) Effects sizes and *P*-values: what should be reported and what should be replicated. *Psychophysiology* 33:157-183.
- Hasselmo ME, McGaughy J (2004) High acetylcholine levels set circuit dynamics for attention and encoding and low acetylcholine levels set dynamics for consolidation. *Progr Brain Res* 145:201-231.
- Herberg LJ, Stephens DN, Franklin KB (1976) Catecholamines and self-stimulation: evidence suggesting a reinforcing role for noradrenaline and a motivating role of dopamine. *Pharmacol Biochem Behav* 4: 575-582.
- Himmelheber AM, Sarter M, Bruno JP (1997) Operant performance and cortical acetylcholine release: role of response rate, reward density, and non-contingent stimuli. *Cogn Brain Res* 6:23-36.
- Himmelheber AM, Sarter M, Bruno JP (2000) Increases in cortical acetylcholine release during sustained attention performance in rats. *Cogn Brain Res* 9:313-325.
- Himmelheber AM, Sarter M, Bruno JP (2001) The effects of manipulations of attentional demand on cortical acetylcholine release. *Cogn Brain Res* 12:353-370.

- McGaughy J, Sarter M (1995) Behavioral vigilance in rats: task validation and effects of age, amphetamine, and benzodiazepine receptor ligands. *Psychopharmacology (Berl)* 117:340-357.
- McGaughy J, Kaiser T, Sarter M (1996) Behavioral vigilance following infusions of 192 IgG-saporin into the basal forebrain: selectivity of the behavioral impairment and relation to cortical AChE-positive fiber density. *Behav Neurosci* 110:247-265.
- McGaughy J, Everitt BJ, Robbins TW, Sarter M (2000) The role of cortical cholinergic afferent projections in cognition: impact of new selective immunotoxins. *Behav Brain Res* 115:251-263.
- McGaughy J, Dalley JW, Morrison CH, Everitt BJ, Robbins TW (2002) Selective behavioral and neurochemical effects of cholinergic lesions produced by intrabasalis infusions of 192 IgG-saporin on attentional performance in a five-choice serial reaction time task. *J Neurosci* 22:1905-1913.
- Moore H, Sarter M, Bruno JP (1995) Bidirectional modulation of cortical acetylcholine efflux by infusion of benzodiazepine receptor ligands into the basal forebrain. *Neurosci Lett* 189:31-34.
- Moore H, Fadel J, Sarter M, Bruno JP (1999) Role of accumbens and cortical dopamine receptors in the regulation of cortical acetylcholine release. *Neuroscience* 88:811-822.
- Neigh G, Arnold MH, Rabenstein R, Sarter M, Bruno JP (2004) Neuronal activity in the nucleus accumbens is necessary for performance-related increases in cortical acetylcholine release. *Neuroscience* 123:635-645.
- Neigh-McCandless G, Kravitz BA, Sarter M, Bruno JP (2002) Stimulation of cortical acetylcholine release following blockade of ionotropic glutamate receptors in nucleus accumbens. *Eur J Neurosci* 16:1259-1266.
- Nelson CL, Sarter M, Bruno JP (2005) Prefrontal modulation of posterior parietal acetylcholine release: prefrontal glutamatergic and cholinergic mechanisms. *Neuroscience* (in press).
- Passetti F, Dalley JW, O'Connell MT, Everitt BJ, Robbins TW (2000) Increased acetylcholine release in the rat medial prefrontal cortex during performance of a visual attentional task. *Eur J Neurosci* 12:3051-3058.
- Paxinos G, Watson C (1988) *The rat brain in stereotaxic coordinates*, 4th edn. San Diego, CA: Academic Press.
- Pecina S, Berridge KC (2000) Opioid site in nucleus accumbens shell mediates eating and hedonic 'liking' for food: map based on microinjection Fos plumes. *Brain Res* 863:71-86.
- Pepeu G, Giovannini MG (2004) Changes in acetylcholine extracellular levels during cognitive processes. *Learn Mem* 11:21-27.
- Rasmusson DD, Szerb IC, Jordan JL (1996) Differential effects of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and *N*-methyl-D-aspartate receptor antagonists applied to the basal forebrain on cortical acetylcholine release and electroencephalogram desynchronization. *Neuroscience* 72:419-427.
- Routtenberg A (1972) Intracranial chemical injection and behavior: a critical review. *Behav Biol* 7:601-641.
- Sarter M, Bruno JP (2002) The neglected constituent of the basal forebrain corticopetal projection system: GABAergic projections. *Eur J Neurosci* 15:1867-1873.
- Sarter M, Hasselmo ME, Bruno JP, Givens B (2005) Unraveling the attentional functions of cortical cholinergic inputs: interactions between signal-driven and top-down cholinergic modulation of signal detection. *Brain Res Rev* 48:98-111.
- Thakkar MM, Winston S, McCarley RW (2003) A₁ receptor and adenosinergic homeostatic regulation of sleep-wakefulness: effects of antisense to the A₁ receptor in the cholinergic basal forebrain. *J Neurosci* 23:4278-4287.
- Turchi J, Sarter M (2001) Bidirectional modulation of basal forebrain *N*-methyl-D-aspartate receptor function differentially affects visual attention but not visual discrimination performance. *Neuroscience* 104:407-417.
- Vasey M, Thayer J (1987) The continuing problem of false positives in repeated measures ANOVA in psychophysiology: a multivariate solution. *Psychophysiology* 24:479-486.
- Zaborszky L, Gaykema RP, Swanson DJ, Cullinan WE (1997) Cortical input to the basal forebrain. *Neuroscience* 79:1051-1078.
- Zar JH (1974) *Biostatistical analysis*. Englewood Cliffs, NJ: Prentice Hall.