# Basal Forebrain Glutamatergic Modulation of Cortical Acetylcholine Release

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*ABSTRACT*      The mediation of cortical ACh release by basal forebrain glutamate receptors was studied in awake rats fitted with microdialysis probes in medial prefrontal cortex and ipsilateral basal forebrain. Repeated presentation of a stimulus consisting of exposure to darkness with the opportunity to consume a sweetened cereal resulted in a transient increase in cortical ACh efflux. This stimulated release was dependent on basal forebrain glutamate receptor activity as intrabasalis perfusion with the ionotropic glutamate receptor antagonist kynurenate (1.0 mM) markedly attenuated darkness/cereal-induced ACh release. Activation of AMPA/kainate receptors by intrabasalis perfusion of kainate (100 µM) was sufficient to increase cortical ACh efflux even under basal (nonstimulated) conditions. This effect of kainate was blocked by coperfusion with the antagonist DNQX (0.1–5.0 mM). Stimulation of NMDA receptors with intrabasalis perfusion of NMDA (50 or 200 µM) did not increase basal cortical ACh efflux. However, perfusion of NMDA in rats following exposure to the darkness/cereal stimulus resulted in a potentiation of both the magnitude and duration of stimulated cortical ACh efflux. Moreover, intrabasalis perfusion of the higher dose of NMDA resulted in a rapid increase in cortical ACh efflux even before presentation of the darkness/cereal stimulus, suggesting an anticipatory change in the excitability of basal forebrain cholinergic neurons. These data demonstrate that basal forebrain glutamate receptors contribute to the stimulation of cortical ACh efflux in response to behavioral stimuli. The specific roles of basal forebrain glutamate receptor subtypes in mediating cortical ACh release differ and depend on the level of activity of basal forebrain cholinergic neurons. **Synapse 39: 201–212, 2001.**   © 2001 Wiley-Liss, Inc.

## INTRODUCTION

The functions of corticopetal projections arising from the substantia innominata, the nucleus basalis of Meynert and the horizontal limb of the diagonal band (collectively termed "basal forebrain"; Mesulam et al., 1983; Eckenstein et al., 1988; Bigl et al., 1982) constitute a main research subject in cognitive neuroscience. These projections terminate in all cortical areas and layers, suggesting that this system gates cortical information processing. This hypothesis is supported by studies on the role of acetylcholine (ACh) in cortical sensory information processing and cognitive functions (see Everitt and Robbins, 1997; Sarter and Bruno, 1997). Cortical cholinergic inputs mediate the early steps of information processing, ranging from the detection, selection, and processing of stimuli (sustained and selective attention) to the regulation and allocation of processing resources (divided attention; Turchi and Sarter, 1997). Accordingly, loss or inhibition of cortical cholinergic inputs is predicted to result in attentional

impairments which evolve into major impairments in cognition and dementia. Conversely, persistent disinhibition of the activity of these inputs may mediate the pathological overprocessing of selected or even irrelevant stimuli, thereby contributing to psychiatric disorders (Sarter and Bruno, 1999).

The present studies focused on the regulation of basal forebrain cholinergic corticopetal projections by glutamate receptor ligands. Glutamatergic afferents to basal forebrain cholinergic corticopetal neurons arise from cortical and amygaloid areas (Gaykema et al., 1991; Zaborszky et al., 1997; Haring and Wang, 1986; Carnes et al., 1990). Telencephalic glutamatergic projections may contact basal forebrain cholinergic neu-

rons (Zaborszky and Cullinan, 1992). Receptors for both α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainic acid (AMPA/KA) and N-methyl-D-aspartate (NMDA), the two major classes of ionotropic excitatory amino acid receptors, are present in basal forebrain (Martin et al., 1993; Page and Everitt, 1995).

While the studies outlined above demonstrate an anatomical substrate for a glutamatergic influence on basal forebrain cholinergic neurons, studies utilizing the administration of glutamatergic agonists into this region have largely focused on effects secondary to the excitotoxic consequences of such manipulations (e.g., Page et al., 1993; Weiss et al., 1994). In contrast, studies investigating the modulatory role of glutamatergic inputs on corticopetal neurons are scarce. Basal forebrain administration of glutamate excites cholinergic neurons in this area (Lamour et al., 1986) and increases cortical high-affinity choline uptake (Wenk, 1984) and cortical ACh efflux (Kurosawa et al., 1989). Conversely, intrabasalis administration of the competitive NMDA antagonist chlorophenylpiperazine (CPP) decreases cortical ACh efflux in anesthetized rats (Giovannini et al., 1997). Intrabasalis glutamatergic transmission has also been implicated in pedunculopontine tegmental (PPT)-stimulation-induced increases in cortical ACh release and EEG desynchronization (Rasmusson et al., 1994, 1996).

The present experiments utilized dual-probe microdialysis techniques in awake rats to answer several questions related to the glutamatergic regulation of the basal forebrain–cortical cholinergic system. First, we determined the contributions of basal forebrain glutamate receptor activity to the well-established ability of an environmental stimulus, exposure to darkness and the opportunity to consume a cereal treat, to increase cortical ACh release (Fadel et al., 1996; Moore et al., 1992, 1993, 1995a). Specifically, the necessity of glutamate receptor activity to this stimulated release was assessed by measuring the effects of intrabasalis perfusion of the nonselective ionotropic antagonist kynurenate on ACh efflux in medial prefrontal cortex (mPFC) following exposure to darkness/cereal. The mPFC site was selected for ACh dialysis in order to preserve comparability with previous data on the regulation of cortical ACh efflux (e.g., Moore et al., 1999; Arnold et al., 2000) and for practical reasons as placement of the guides into the mPFC did not interfere with the basal forebrain guides used to perfuse glutamatergic drugs. As predicted by the relatively diffuse anatomical organization of basal forebrain corticopetal cholinergic projections (Sarter and Bruno, 1997), several previous studies (e.g., Himmelheber et al., 1998) indicated that the regulation of cortical ACh release does not significantly differ between cortical areas (see also the discussion in Moore et al., 1999). Thus, the present results may generalize to other cortical areas.

Second, we determined the sufficiency of basal forebrain glutamate receptor activity to stimulate cortical ACh release under standard environmental conditions by measuring efflux following intrabasalis perfusions of kainate or NMDA. Finally, given the speculation that the effects of NMDA receptor activation on cortical ACh release might be optimal under conditions of basal forebrain excitation, we determined if the effects of local perfusion of NMDA interacted with the effects of the environmental (exposure to darkness/cereal) stimulus on cortical ACh release.

## MATERIALS AND METHODS
### Subjects, habituation, and the darkness/cereal stimulus

Young adult (3–7 months of age) male Fischer 344/Brown Norway F1 hybrid rats were used for all experiments. This strain, age, and gender were chosen based on extensive, relevant pharmacological and behavioral data generated with these rats in our and other laboratories. Animals were housed on a 12:12 hr light:dark cycle (lights on at 0600) with food and water available ad libitum. All housing, surgery, experimentation, and euthanasia were performed in accordance with Ohio State University Animal Care and Use Committee-approved protocols and U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

For 10 consecutive days prior to surgery, animals were handled and allowed to habituate to the microdialysis testing environment, the animal containment portion of which consisted of clear, plastic parabolic bowls (35 cm ht. × 38 cm dia.) lined with sanitized wood chip bedding. During this time, one group of animals received daily exposure to sudden darkness in the microdialysis testing room paired with presentation of a piece of fruit-flavored cereal. In certain cases, mild food deprivation (weight loss not exceeding 5% of free-feed weight) was required to motivate the animals to initially eat the novel food. Once the cereal was consumed, animals vigorously approached and consumed the cereal in future sessions, even after the resumption of free-feeding in their home cages. In all cases, by the end of the 10-day exposure period the latency to approach and consume the cereal was less than 30 secs. This manipulation has repeatedly been demonstrated to increase cortical ACh efflux (Moore et al., 1993; Fadel et al., 1996) and was used simply as a means of stimulating the basal forebrain cholinergic system in order to evaluate the contributions of intrabasalis glutamate receptors to cortical ACh release. A second group of animals received an equivalent amount of handling and habituation during the 10-day period prior to surgery, but were not exposed to the darkness/cereal stimulus during the habituation period or during dialysis testing.

## Guide cannula implantation

On the 11th day following the onset of habituation, animals were anesthetized with sodium pentobarbital (60.0 mg/kg, i.p.) and stereotaxically implanted with microdialysis guide cannulae (0.65 mm o.d.; Bioanalytical Systems (BAS), West Lafayette, IN) in medial prefrontal cortex (mPFC) and the ventral pallidum/substantia innominata (VP/SI) region of the basal forebrain. The mPFC cannula was placed in the left hemisphere at the following coordinates: AP +3.0 mm, L +0.8 mm, DV −1.0 mm (from dura). The VP/SI cannula was also placed in the left hemisphere at the following coordinates: AP −2.2 mm, L +2.4 mm, DV −6.3 mm (from dura). The mPFC and VP/SI cannulae were angled 10° rostrally and 15° caudally, respectively. All coordinates, relative to bregma, were taken from the atlas of Paxinos and Watson (1985). The positions of the cannulae were fixed with dental cement and skull screws. At the conclusion of surgery, animals were given a prophylactic dose of the antibiotic amoxycillin (30,000 U, i.m.) and placed in single housing tubs in the vivarium. Handling, habituation, and (for one of the two groups) daily exposure to the darkness/cereal stimulus continued for the 3 days between surgery and the first microdialysis session. No gross behavioral changes, including the animals' latency to consume the fruit-flavored cereal, were observed following surgery.

## Microdialysis procedures

The timeline for each microdialysis session was held constant. Animals were placed in the microdialysis bowls between 0800–0900. The stainless steel stylets used to maintain the patency of the guide cannulae were removed and replaced with concentric microdialysis probes (BAS) with semipermeable probe membrane tips (0.5 mm o.d.; nominal MW cutoff = 15,000) which extended 2.0 mm beyond the end of the guide cannula. The probes were perfused at 1.25 μl/min with an artificial cerebrospinal fluid (aCSF; pH = 6.9 ± 0.1) composed of an aqueous solution of the following (in mM): NaCl 126.5, NaHCO$_3$ 27.5, KCl 2.4, Na$_2$SO$_4$ 0.5, KH$_2$PO$_4$ 0.5, CaCl$_2$ 1.1, MgCl$_2$ 0.8, and glucose 4.9. The acetylcholinesterase inhibitor neostigmine bromide (0.5 μM; Sigma Chemical, St. Louis, MO) was added to the cortical aCSF to promote recovery of detectable levels of basal ACh with concentric dialysis probes. No dialysates were collected for the first 3 h following probe insertion to allow for the reestablishment of impulse-dependent ACh efflux in the zone surrounding the cortical probe (Moore et al., 1992). Following this delay, collection of 15-min samples was initiated beginning with four consecutive baseline measurements. After the last baseline collection, the line leading to the basal forebrain probe was switched to a drug-containing aCSF (or, in the case of vehicle sessions, simply to another syringe containing drug-free aCSF). Beginning 15 min after this switch (to allow the drug to reach the

perfusion zone of the basal forebrain probe), several additional cortical dialysates were collected. Immediately following the second of these postdrug collections, some animals were exposed to the darkness/cereal stimulus, thus allowing for assessment of drug effects on cortical ACh efflux prior to and in interaction with the environmental stimulus in these animals. At the end of each session, dialysis probes were removed and replaced with stylets and the animals were returned to their home cages. All animals received 3–4 dialysis sessions, with a drug-free nondialysis day between each session. Drug treatments in each experiment were counterbalanced across dialysis sessions. The validity of repeated-sessions microdialysis designs for measurement of both cortical (Moore et al., 1995b) and striatal (Johnson and Bruno, 1995) ACh efflux has been previously demonstrated. At the conclusion of the final dialysis session, a subset of animals received intracranial administration (via either the mPFC or basal forebrain probe) of the fluorescent retrograde tracer Fluoro-gold to visualize probe placements and provide a rough estimate of the perfusion zone (FluoroChrome, Englewood, CO; 15 min perfusion with 0.1% solution).

Within 3 days of their final dialysis session, animals were deeply anesthetized with sodium pentobarbital and sacrificed by transcardial perfusion with heparinized saline followed by 10% formalin. Fixed brains were removed from the cranial cavity, postfixed for an additional 24 h, and transferred to a 30% sucrose phosphate buffer solution for cryoprotection. Sections (45 μm) through mPFC and VP/SI were obtained with a freezing microtome, mounted on slides, and Nissl-stained (cresyl violet) for verification of probe placement.

## Quantitation of acetylcholine

Dialysates were stored at −80°C until analysis by high-performance liquid chromatography (HPLC) utilizing modifications of previously published methods (Potter et al., 1983). Briefly, 12.5 μl of each dialysate were loaded onto the carbon-polymer analytical column (150 × 1.0 mm; BAS) in interaction with a mobile phase (pH 8.45) consisting of 35.0 mM NaH$_2$PO$_4$, 10.0 mM NaCl, and 10.0 mM Na$_2$EDTA. The microbicide Kathon (0.01%) was added to prevent bacterial contamination. Following separation, postcolumn derivitization of ACh and choline was achieved by an additional column segment containing covalently bound acetylcholinesterase and choline oxidase, generating stoichiometric quantities of hydrogen peroxide, which was further broken down and detected on a 3.0 mm glassy carbon "wired" electrode (0 mV applied potential) coated with a horseradish peroxidase osmium polyvinylpyridine solution (Huang et al., 1995). ACh levels in each dialysate were assessed by comparison with a four-point external standard curve bounding the expected range of in vivo dialysate values. The detection limit of this method was 10.0 fmol ACh/injection.

## Statistical analysis

Basal cortical ACh efflux for each animal in each dialysis session was expressed as the median of the four baseline collections for that session. Efflux following behavioral and/or pharmacological manipulations was then expressed as a percent change from the median baseline. Efflux measurements were not corrected for probe recovery. However, a representative sampling of in vitro recovery measurements (obtained by dialyzing at the in vivo flow rate from a probe placed in a 0.1 $\mu$M ACh 0.5 $\mu$M choline solution) from these and previous experiments utilizing the same type of probes suggests a reliable recovery rate of 10–15%.

Data were subjected to analysis of variance (ANOVA) as a test for significant main effects and interactions, with DRUG (or DOSE) and TIME as within-subjects variables and, where relevant, ENVIRONMENTAL CONDITION as a between-subjects variable. Significance was defined as $P \leq 0.05$. Significant interactions from multifactor ANOVAs were broken down into multiple one-way ANOVAs as a first step toward locating the source(s) of significant F-statistics. Planned comparisons, consisting of paired or independent $t$-tests measured against a modified Bonferroni-corrected $\alpha$, were then conducted on the means contributing to the significant one-way ANOVAs. This correction places constraints on the performance of an excessive number of comparisons, while simultaneously not being overly stringent on a smaller number of comparisons, thus achieving a balance between Type I and Type II errors (Keppel, 1991). The modified Bonferroni-corrected $\alpha$ ($\alpha_{MB}$) is calculated as $\alpha_{MB} = \text{d.f.}_n (\alpha)/pc$, where d.f.$_n$ = degrees of freedom in the numerator (error term), $\alpha$ = the uncorrected $\alpha$-value (0.05), and pc refers to the actual number of planned comparisons performed. In cases where the number of planned comparisons did not exceed the d.f.$_n$, an uncorrected $\alpha$ value of 0.05 was used, and thus no $\alpha_{MB}$ was reported. All statistical tests were performed using SPSS for Windows software (V 7.5, SPSS, Chicago, IL).

## Experiments
### Experiment 1: behaviorally stimulated cortical ACh efflux: dependence on basal forebrain glutamatergic transmission

All animals used in this experiment ($n = 8$) were exposed to the darkness/cereal stimulus as described in Methods. Four microdialysis sessions were conducted, with a nondialysis day between each session. Following the last baseline dialysate collection in each of these sessions, the inlet line of the basal forebrain probe was switched to another drug-free aCSF, or an aCSF containing the nonselective ionotropic glutamate receptor antagonist kynurenate (1.0 mM). This dose of kynurenate, perfused into the basal forebrain, is within the range of doses shown by Rasmusson et al. (1994) to

attenuate the increase in cortical ACh efflux resulting from stimulation of the PPT in anesthetized rats. Perfusion of the basal forebrain with kynurenate or drug-free aCSF continued for six additional 15-min collection periods. Between the third and fourth of these collections, animals received the darkness/cereal stimulus (two dialysis sessions). Thus, the two dialysis sessions consisted of the darkness/cereal stimulus crossed with the presence or absence of intrabasalis kynurenate and were conducted in a pseudo-randomized order.

The initial statistical analysis was designed to assess the stability of baseline cortical ACh efflux and to determine whether repeated dialysis perfusions affected basal efflux. A two-way ANOVA with TIME (four baseline collections) and SESSION (two sessions) as within subject factors.

The second statistical analysis determined the ability of the darkness/cereal stimulus to stimulate cortical ACh efflux in the absence of intrabasalis kynurenate. A one-way TIME ANOVA using the last baseline collection and the three postmanipulation timepoints was conducted. The effects of intrabasalis kynurenate on basal efflux were then investigated by a DRUG (0 or 1.0 mM kynurenate) $\times$ TIME (three collections after baseline but prior to darkness/cereal) ANOVA. Finally, the effects of kynurenate on stimulated efflux were similarly assessed using the three postdarkness/cereal timepoints. Breakdown of significant interactions in two-way ANOVAs and planned comparisons utilizing a modified Bonferroni-corrected alpha ($\alpha_{MB}$) for the determination of significant $P$-values were conducted as described in Methods.

### Experiment 2: stimulation of cortical ACh efflux by intrabasalis perfusions of AMPA/KA receptor agonists

This experiment determined the ability of intrabasalis perfusion of KA to stimulate cortical ACh efflux under normal laboratory conditions (i.e., no darkness/cereal, $n = 8$). Each of these subjects underwent four microdialysis sessions, administered in counterbalanced order. Each session began with the collection of four 15-min baseline dialysates, followed by a switch of the inlet line to the basal forebrain probe to an aCSF solution containing the AMPA/KA antagonist DNQX at one of four concentrations (0.0, 0.1, 1.0, or 5.0 mM). Following a 15-min delay to account for the dead volume in the dialysis swivel channel, inlet lines, and probe, four additional cortical dialysates were collected for assessment of the effects of intrabasalis DNQX on basal ACh efflux. The basal forebrain probe was then switched again to an aCSF containing the same concentration of DNQX plus 100 $\mu$M KA. Again, following a 15-min delay, four additional cortical dialysates were collected to determine the ability of KA to stimulate cortical ACh efflux and whether this effect was selec-

tive for the AMPA/KA receptor. The single dose of KA was based on its ability, in pilot studies, to produce reliable increases in cortical ACh efflux and is comparable to doses shown to stimulate hippocampal ACh efflux when administered via a medial septal dialysis probe (Moore et al., 1994).

As in Experiment 1, the initial statistical analysis was designed to assess the stability of baseline cortical ACh efflux and to determine whether repeated dialysis perfusions affected basal efflux. A two-way ANOVA with TIME (four baseline collections) and SESSION (two sessions) as within-subject factors.

The ability of intrabasalis KA to increase cortical ACh efflux was first assessed in a one-way ANOVA (TIME as the factor) incorporating the last pre- and four postintrabasalis KA timepoints in the absence of DNQX (i.e., the 0.0 mM, or vehicle, DNQX session). Second, the effect of intrabasalis DNQX on basal cortical ACh efflux was determined by a two-way ANOVA with DNQX DOSE (0.0, 0.1, 1.0, 5.0 mM) and TIME (four post-DNQX/pre-KA collections) as factors. Finally, the ability of DNQX to block KA-stimulated cortical ACh efflux was assessed by a two-way ANOVA with DNQX DOSE and TIME (four post-KA collections) as factors.

## Experiment 3: effects of intrabasalis NMDA on cortical ACh efflux: interactions with environmental stimulation

Subjects in this experiment were divided equally between environmentally stimulated (i.e., darkness/cereal, $n = 6$) and nonstimulated ($n = 6$) subgroups. Exposure to the darkness/cereal stimulus was exactly as described in Methods. Each animal received three dialysis sessions (one for each dose of NMDA) in counterbalanced order. In each session, following the last (fourth) baseline cortical ACh collection, the syringe perfusing the intrabasalis probe was switched to one containing 0, 50, or 200 μM NMDA. This range of NMDA doses encompasses a concentration demonstrated to increase hippocampal ACh efflux when perfused into the medial septum (Moor et al., 1994) and is well below the minimum concentrations of perfused NMDA (10 mM) needed to produce excitotoxic effects (Vanicky et al., 1998). Collection of cortical dialysates resumed following a 15-min delay and continued for an additional six 15-min collection intervals. Immediately prior to the beginning of the third post-NMDA collection period, entrained animals received the darkness/cereal stimulus.

As in the previous experiments, the initial statistical analysis was designed to assess the stability of baseline cortical ACh efflux (pmol/dialysis sample) and to determine whether repeated dialysis perfusions affected basal efflux differentially in the two environmental conditions. Thus, we conducted a three-way ANOVA with TIME (four baseline collections) and SESSION (three sessions) as within-subject factors and ENVIRONMENTAL CONDITION (standard vs. darkness/cereal) as a between-subject factor.

Within the nonstimulated animals, the effect of intrabasalis NMDA on cortical ACh efflux was assessed by a NMDA DOSE (0, 50, 200 μM) × TIME (six post-NMDA collections) ANOVA. Within the stimulated group of animals, three separate ANOVAs were performed to investigate different aspects of the manipulations. First, to establish the darkness/cereal effect on cortical ACh efflux, a one-way TIME (last pre- and four postdarkness/cereal collections) ANOVA was performed on the 0 μM NMDA session. Second, to assess the effects of intrabasalis NMDA on basal cortical ACh efflux, a NMDA DOSE × TIME (two post-NMDA, predarkness/cereal collections) ANOVA was performed. Finally, to assess the possibility of an interaction between intrabasalis NMDA and the magnitude of the darkness/cereal-stimulated cortical ACh efflux, an NMDA DOSE × TIME (four postdarkness/cereal collections) ANOVA was performed.

## RESULTS
### Experiment 1: environmentally stimulated cortical ACh efflux: dependence on glutamatergic transmission within the basal forebrain

Representative placements of the probes located in the medial prefrontal cortex and the basal forebrain are shown in Figure 1.

Basal cortical ACh efflux (mean ± SEM) was 0.268 ± 0.54 and 0.169 ± 0.049 pmol/sample for the first and second dialysis sessions, respectively. There was no significant difference between these two baselines (SESSION, $F_{1,7} = 2.842$, $P = 0.136$). In addition, basal efflux was stable over baselines (TIME, $F_{3,21} = 0.866$, $P = 0.474$; TIME × SESSION, $F_{3,21} = 0.190$, $P = 0.902$). These nonsignificant effects allow for subsequent analyses on the effects of drugs and environmental stimulation to be expressed as percent change from baseline.

Figure 2 shows the effects of the darkness/cereal stimulus, in the presence or absence of intrabasalis kynurenate, on cortical ACh efflux. First, the darkness/cereal stimulus, in the absence of intrabasalis kynurenate, transiently enhanced cortical ACh efflux ($F_{3,21} = 12.887$; $P < 0.001$). Planned paired comparisons indicated that this effect was driven by ACh efflux being significantly higher at the first darkness/cereal timepoint (60 min) compared to the preceding collection (45 min, $t_7 = 5.633$; $P = 0.001$; $\alpha_{MB} = 0.05$). No other comparisons were significant. Second, intrabasalis perfusion of kynurenate diminished basal (i.e., prior to darkness/cereal) ACh efflux (DRUG $F_{1,7} = 10.638$; $P = 0.014$), although inspection of Figure 1 suggests that this reduction is largely attributable to the unexpected trend toward an increase in
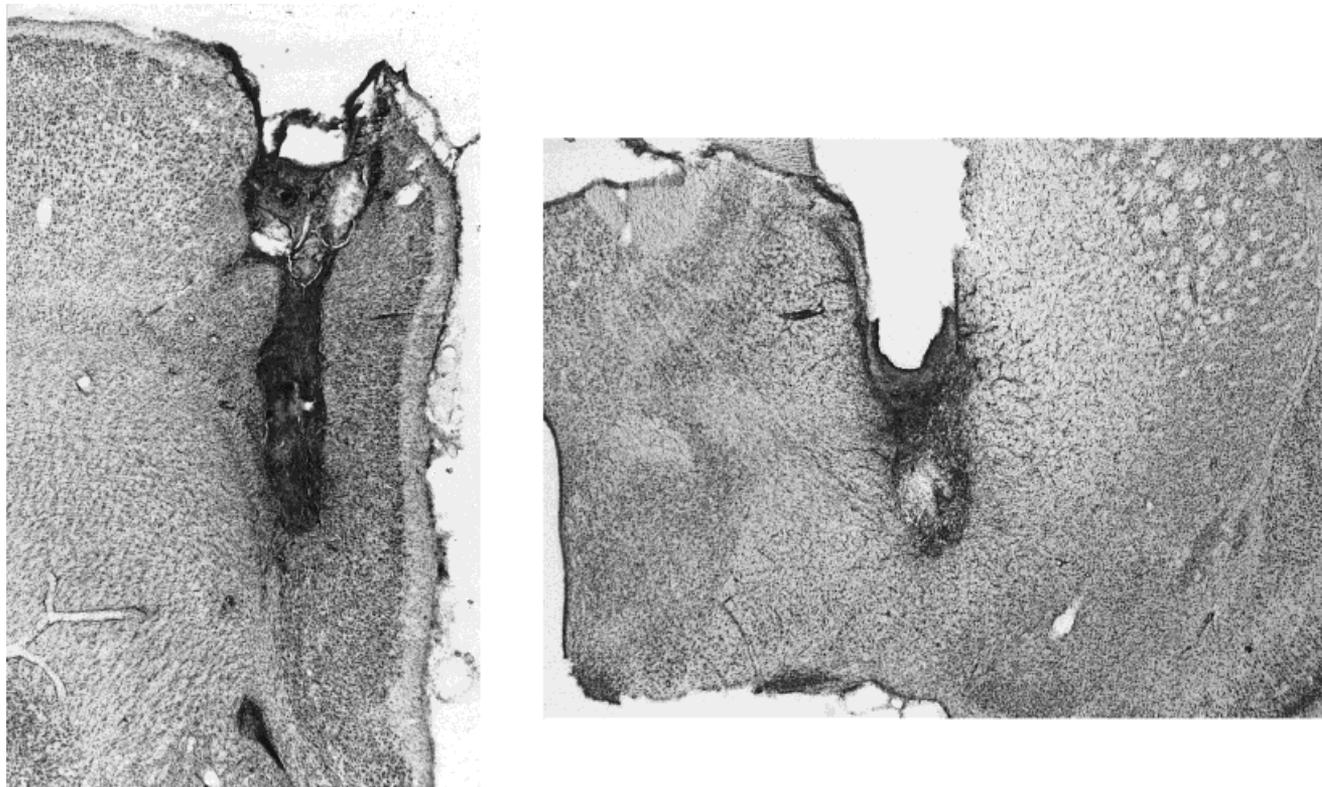
Fig. 1. Representative placements of the probes in the medial prefrontal cortex (left; magnification 6.4×) and basal forebrain (right; magnification 5×; Nissl-stained coronal sections). In both microphotographs, the placement of the guide cannula and the damage produced by the probe can be clearly dissociated. The tip of the prefrontal guide cannula was located in the anterior cingulate cortex while the probe penetrated the lower layers of the prelimbic area (Area 25). The probe in the basal forebrain (right) was located in the ventral portion of the medial globus pallidus, that is, the region of the magnocellular cholinergic neurons of the nucleus basalis of Meynert and, more ventrally, in the substantia innominata.

basal efflux in vehicle-treated controls at 15 min. This is supported by the lack of a significant TIME effect in a one-way ANOVA (last baseline, 15, 30, and 45 min time points) on the kynurenate session ($F_{3,21} = 1.959$, $P = 0.151$). The kynurenate effect at these predarkness/cereal time points may thus be the result of a suppression of a nonsignificant anticipatory trend toward increased cortical ACh efflux prior to the darkness/cereal stimulus. This conclusion is also supported by the fact that there were also no significant overall effects of TIME ($F_{2,14} = 0.557$; $P > 0.05$) or a DRUG × TIME interaction ($F_{2,14} = 0.207$; $P > 0.05$). Finally, kynurenate markedly attenuated the ability of darkness/cereal to stimulate ACh efflux (DRUG $F_{1,7} = 19.594$; $P = 0.003$). Again, the ANOVA revealed a significant TIME effect ($F_{2,14} = 14.457$; $P < 0.001$), supporting the transient nature of the environmental stimulus, as cortical ACh efflux was elevated at the first darkness/cereal timepoint (60 min) relative to both the second (75 min, $t_7 = 3.975$; $P = 0.005$) and third (90 min, $t_7 = 4.271$; $P = 0.004$) timepoints ($\alpha_{MB} = 0.033$). The ability of intrabasalis kynurenate to block the darkness/cereal-stimulated increase in cortical ACh efflux was also supported by a DRUG × TIME interaction ($F_{2,14} = $
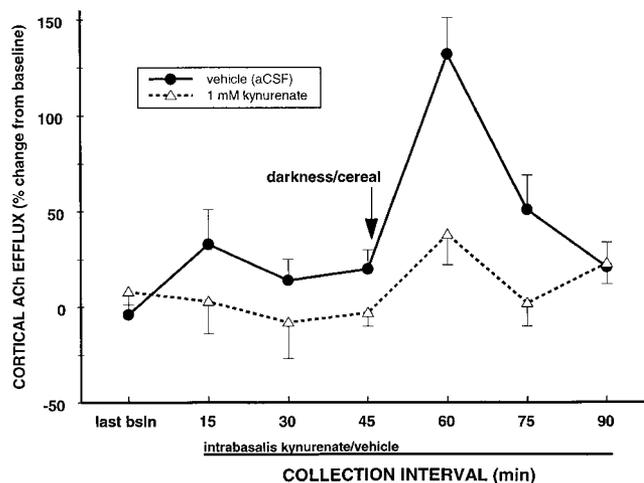


Fig. 2. Mean (± SEM) cortical ACh efflux, expressed as percent change from baseline, following exposure to the darkness/cereal stimulus. All animals ($n = 8$) were implanted with dual microdialysis probes in mPFC and ipsilateral basal forebrain. Each animal received a control vehicle perfusion (aCSF) or the nonselective ionotropic glutamate receptor antagonist kynurenate (1.0 mM) directly into basal forebrain in two counterbalanced dialysis sessions. Exposure to the darkness/cereal stimulus resulted in a transient increase in cortical ACh efflux. This enhanced ACh efflux was markedly attenuated by intrabasalis perfusion of kynurenate.

5.925; $P = 0.014$) resulting from cortical ACh efflux being greater at the first darkness/cereal timepoint (60 min) in the vehicle session relative to the kynurenate session ($t_7 = 5.443$; $P = 0.001$; $\alpha_{MB} = 0.033$).

## Experiment 2: stimulation of cortical ACh efflux by intrabasalis perfusions of AMPA/KA receptor agonists

The results of Experiment 1 demonstrate the necessity of glutamatergic transmission within the basal forebrain for the full expression of stimulated cortical ACh efflux following an environmental stimulus. However, little is known regarding the sufficiency of glutamate receptor activation for stimulation of cortical ACh efflux. Basal forebrain cholinergic neurons respond to glutamatergic stimulation by increasing their firing rates (Lamour et al., 1986) and increasing release of ACh within terminal regions, such as the cerebral cortex and hippocampus (Kurosawa et al., 1989). Nonetheless, the subtype-specific glutamate receptor contributions to these effects remain unclear. Thus, Experiment 2 was designed to test the ability of intrabasalis administration of the AMPA/KA agonist kainic acid (KA) to increase cortical ACh efflux.

Basal cortical ACh efflux (mean $\pm$ SEM) for the four microdialysis sessions was $0.307 \pm 0.091$, $0.348 \pm 0.124$, $0.167 \pm 0.048$, and $0.231 \pm 0.058$ pmol/sample. A two-way ANOVA revealed that basal efflux was comparable among the four sessions (SESSION, $F_{3,21} = 1.077$, $P = 0.380$). Moreover, basal efflux was stable over time across the four dialysis sessions (TIME, $F_{3,21} = 1.112$, $P = 0.366$, TIME $\times$ SESSION, $F_{9,63} = 1.399$, $P = 0.208$). Thus, subsequent analyses of the effects of drugs on cortical ACh efflux were conducted on data expressed as a percent change from these baseline values.

Figure 3 summarizes the effects of intrabasalis perfusion of 100 $\mu$M KA, in the presence of 0–5.0 mM DNQX, on cortical ACh efflux. In the absence of DNQX, KA increased cortical ACh efflux, as indicated by a significant effect of TIME over the last pre- and fourth post-KA periods in the 0 mM DNQX session ($F_{4,28} = 12.062$; $P < 0.001$). This effect resulted from ACh efflux being significantly elevated, relative to the last pre-KA timepoint, during the second (90 min, $t_7 = 5.634$; $P = 0.001$), 3rd (105 min, $t_7 = 5.033$; $P = 0.002$), and 4th (120 min, $t_7 = 4.115$; $P = 0.004$) collections following the onset of KA perfusion. The specificity of this effect was demonstrated by the ability of the AMPA/KA antagonist DNQX to prevent this increase. The main effect of DOSE ($F_{3,21} = 8.206$; $P = 0.001$) in this analysis was the result of cortical ACh efflux being significantly greater in the absence of DNQX compared to 1.0 mM DNQX ($t_7 = 3.203$; $P = 0.015$) and also greater ACh efflux in the presence of 0.1 mM DNQX compared to 1.0 mM ($t_7 = 3.232$; $P = 0.014$) or 5.0 mM ($t_7 = 5.350$; $P = 0.001$) DNQX
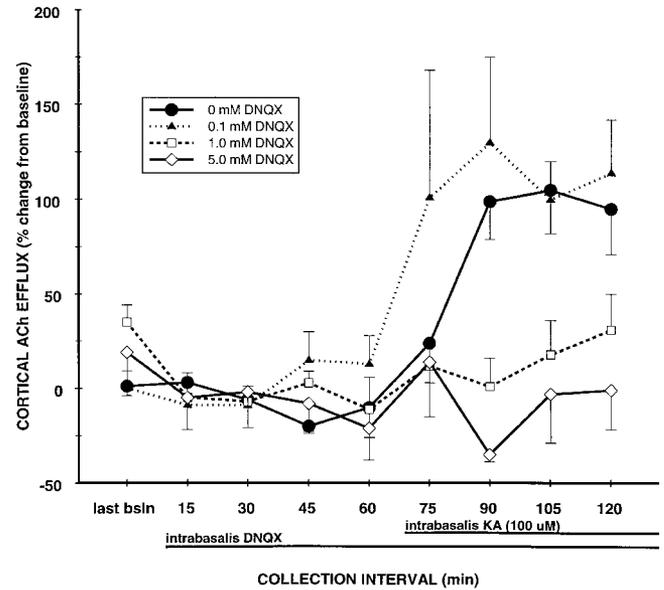


Fig. 3. Mean ($\pm$ SEM) cortical ACh efflux, expressed as percent change from baseline, in nonstimulated rats following intrabasalis perfusion of kainate (KA, 100 $\mu$M) with and without coperfusion of the AMPA/kainate receptor antagonist (DNQX). All animals ($n = 8$) were implanted with dual microdialysis probes in mPFC and ipsilateral basal forebrain. After the last baseline (last bsln) animals received intrabasalis perfusions of three different doses of DNQX or simply the aCSF as a control. This perfusion persisted throughout the remainder of the dialysis session. Immediately after the 60-min collection interval, all animals received intrabasalis perfusions of KA for the remainder of the dialysis session. All animal received each of the four treatment combinations in four counterbalanced dialysis sessions. Perfusion of DNQX had no effect on basal cortical ACh efflux. Intrabasalis perfusion of KA stimulated cortical ACh efflux and this increase was blocked by coperfusion with the two higher doses of DNQX.

($\alpha_{MB} = 0.025$). The suppressive effects of DNQX on KA-stimulated ACh efflux persisted throughout the extent of the dialysis session as evidenced by nonsignificant effects of TIME ($F_{3,21} = 0.634$; $P = 0.601$) or the DOSE $\times$ TIME interaction ($F_{9,63} = 0.996$; $P = 0.453$). Finally, the blockade of KA-stimulated cortical ACh efflux by DNQX were not secondary to the effects of the antagonist on basal efflux, as an ANOVA on basal efflux did not reveal any main effects or an interaction (DOSE $F_{3,21} = 0.384$; $P = 0.765$; TIME $F_{3,21} = 0.094$; $P = 0.963$; DOSE $\times$ TIME $F_{9,63} = 1.000$; $P = 0.449$).

## Experiment 3: effects of intrabasalis NMDA on cortical ACh efflux: interactions with environmental stimulation

Because AMPA/KA and NMDA receptors appear, to a large extent, to be colocalized on neurons throughout the brain (Michaelis, 1998), one may predict a qualitatively similar response to stimulation of either of these receptor populations within the basal forebrain region of cortically projecting cholinergic neurons. Thus, on the basis of the results from Experiment 2, one would predict that intrabasalis perfusion of NMDA would be
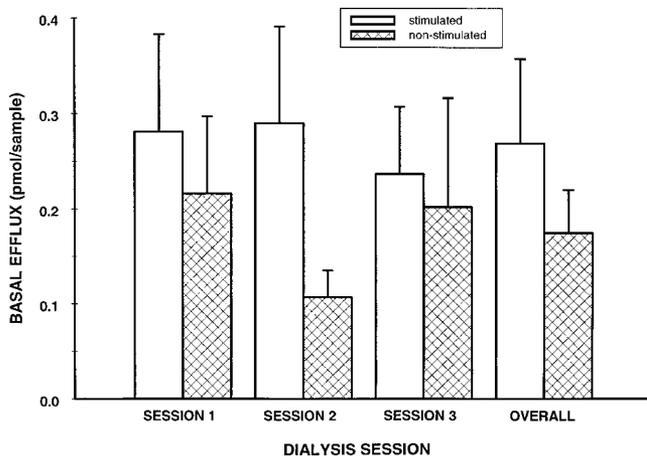
Fig. 4. Mean (± SEM) basal cortical ACh efflux (pmol/dialysate sample) in rats exposed to the darkness/cereal stimulus ($n = 6$) and in nonstimulated controls ($n = 6$). Basal ACh efflux is presented for each of the three microdialysis sessions as well as an overall average. Overall, baseline values of cortical ACh efflux did not differ between the two groups of animals nor was there an interaction with dialysis session.
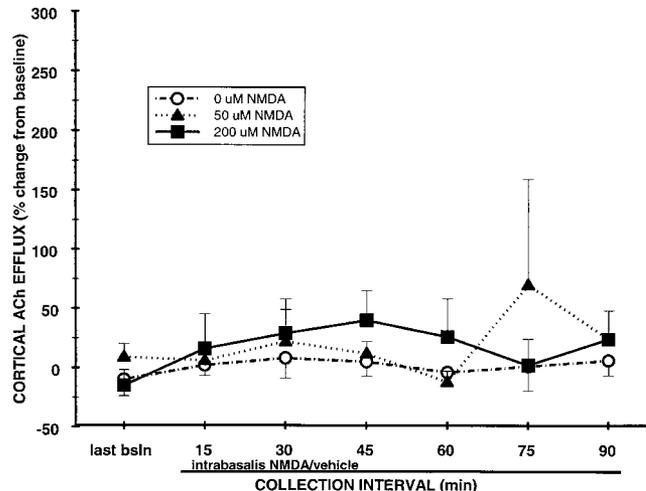


Fig. 5. Mean (± SEM) cortical ACh efflux, expressed as percent change from baseline, in nonstimulated rats following the intrabasalis perfusion of varying doses of NMDA. All animals ($n = 6$) were implanted with dual microdialysis probes in mPFC and ipsilateral basal forebrain. Each animal received a control vehicle perfusion (0 μM NMDA or aCSF) or the NMDA (50 or 200 μM) directly into basal forebrain in three counterbalanced dialysis sessions. NMDA had no effect on cortical ACh efflux.

sufficient to increase basal cortical ACh efflux. However, the numerous regulatory mechanisms associated with the NMDA receptor (e.g., glycine co-agonist binding, polyamine modulation, voltage-dependent $Mg^{++}$ blockade, etc.; for review, see Michaelis, 1998) may represent a molecular substrate for potential behavioral or experience-dependent function of this receptor. While the issue of alterations in behavior as a function of prior drug exposure has been a paradigmatic staple in behavioral pharmacology, the reverse issue (an altered neurochemical response to a novel pharmacological stimulus as a function of behavioral experience) has been less frequently addressed. This latter interaction may hold equally important implications for the understanding of neurochemical correlates of behavioral phenomena. Experiment 3, then, was designed to test the ability of intrabasalis administration of NMDA to increase cortical ACh efflux. The potential for an experience-dependent response to NMDA, suggested from pilot studies, was tested by comparing the effects of intrabasalis NMDA perfusion in animals repeatedly exposed to the darkness/cereal stimulus and in animals not exposed to this stimulus.

Figure 4 summarizes basal efflux values across the three dialysis sessions as well as an overall mean basal efflux are shown for rats in both the darkness/cereal stimulated group and in the nonstimulated group. While the mean basal efflux data are presented for graphical simplicity, the overall three-way ANOVA revealed that baselines were stable (TIME, $F_{3,30} = 0.377$; $P = 0.770$) and did not differ in their stability as a function of session or environmental group (TIME × SESSION × GROUP, $F_{6,60} = 1.075$; $P = 0.324$). This ANOVA also revealed that overall basal efflux did not differ as a function of dialysis session

(SESSION, $F_{2,20} = 2.004$; $P = 0.161$) or environmental group (GROUP, $F_{1,10} = 1.075$; $P = 0.324$). There was, however, a significant interaction between dialysis session and environmental condition (SESSION × GROUP, $F_{2,20} = 3.663$; $P = 0.044$). Posthoc analyses revealed that this was the result of the darkness/cereal group having a higher basal efflux than those in the standard condition on the second dialysis session ($t_{10} = 1.393$, $P = 0.023$, $\alpha_{MB} = 0.033$). This effect was not seen during either of the other two sessions or maintained when the overall means (across sessions) were analyzed.

In marked contrast to the effects seen following kainate perfusion (Fig. 4), intrabasalis administration of NMDA in nonstimulated animals did not affect cortical ACh efflux (Fig. 5; DOSE, $F_{2,10} = 0.374$; $P = 0.697$; TIME, $F_{5,25} = 0.442$; $P = 0.815$; or a DOSE × TIME interaction ($F_{10,50} = 0.522$; $P = 0.867$).

The effects of intrabasalis NMDA administration and darkness/cereal presentation on cortical ACh efflux is shown in Figure 6. First, the darkness/cereal stimulus significantly increased cortical ACh efflux, in the absence of NMDA, as supported by a main effect of TIME ($F_{4,20} = 7.645$; $P = 0.001$). As seen in Experiment 1, only the 1st post-darkness/cereal timepoint (45 min) was elevated relative to the last predarkness/cereal timepoint ($t_5 = 3.667$; $P = 0.014$), illustrating the transient nature of this increase. Second, intrabasalis perfusions of NMDA potentiated darkness/cereal-stimulated cortical ACh efflux as suggested by a significant effect of DOSE ($F_{2,10} = 5.484$; $P = 0.025$). Planned comparisons suggested that this dose dependency resulted from potentiation at the 200 μM
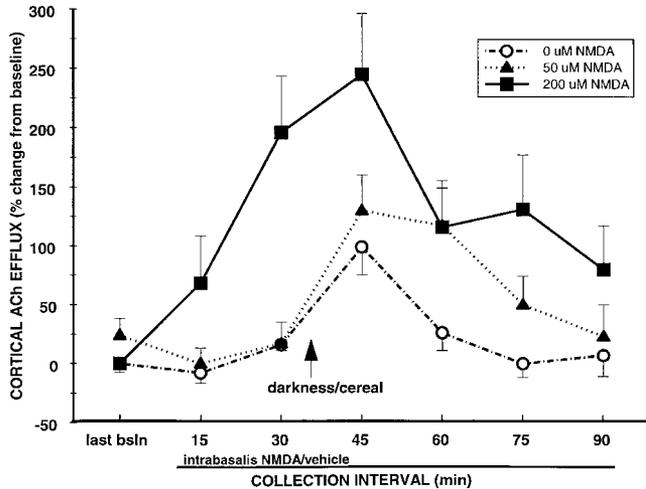
Fig. 6. Mean (± SEM) cortical ACh efflux, expressed as percent change from baseline, in stimulated rats following the intrabasalis perfusion of varying doses of NMDA. All animals (*n* = 6) were implanted with dual microdialysis probes in mPFC and ipsilateral basal forebrain. Each animal received a control vehicle perfusion (0 μM NMDA or aCSF) or NMDA (50 or 200 μM) directly into basal forebrain in three counterbalanced dialysis sessions. Again, exposure to the darkness/cereal stimulus transiently increased cortical ACh efflux during the control (0 μM) session. Perfusion with either dose of NMDA potentiated both the magnitude and duration of the effects of darkness/cereal on ACh efflux. Moreover, intrabasalis perfusion of the higher dose of NMDA (200 μM) produced a marked increase in cortical ACh efflux prior to exposure to the darkness/cereal stimulus.

dose compared with the 0 μM control session ($t_5$ = 2.773; $P$ = 0.039), but this difference did not quite reach significance using the $\alpha_{MB}$ value (0.033). There was also a main effect of TIME ($F_{3,15}$ = 15.718; $P$ < 0.001) resulting from greater ACh efflux at the first (45 min), relative to the third (75 min, $t_5$ = 5.502; $P$ = 0.003) and fourth (90 min, $t_5$ = 6.046; $P$ = 0.002; $\alpha_{MB}$ = 0.025) postdarkness/cereal timepoints, again illustrating the transient nature of the increase following this stimulus. Although Figure 6 descriptively suggests that intrabasalis NMDA prolonged the duration of the darkness/cereal-induced increase in cortical ACh efflux, this was not supported by a significant DOSE × TIME interaction ($F_{6,30}$ = 1.396; $P$ = 0.249).

Finally, NMDA increased basal (i.e., prior to darkness/cereal) cortical ACh efflux in animals repeatedly exposed to the stimulus as evidenced by a significant effect of TIME ($F_{1,5}$ = 12.306; $P$ = 0.017). Planned comparisons suggested that this effect reflected the higher efflux during the second NMDA perfusion timepoint (30 min, $t_5$ = 3.58, $P$ = 0.017). The stimulating effect of NMDA on ACh efflux was dose-dependent (DOSE, $F_{2,10}$ = 7.727; $P$ = 0.009), which appeared to result from greater efflux during the perfusion of 200 μM NMDA relative to 0 μM ($t_5$ = 2.773; $P$ = 0.039), although it was not statistically significant when measured against the $\alpha_{MB}$ value of 0.033. The ability of intrabasalis NMDA to stimulate basal efflux in ani-

mals exposed to darkness/cereal was further revealed by a DOSE × TIME interaction ($F_{2,10}$ = 12.402; $P$ = 0.009), resulting from the near-200% increases at the second NMDA timepoint (30 min) during the 200 μM NMDA session vs. that seen during the 0 μM ($t_5$ = 3.841; $P$ = 0.012) or 50 μM ($t_5$ = 3.416; $P$ = 0.019) sessions ($\alpha_{MB}$ = 0.033).

## DISCUSSION

These experiments resulted in several observations regarding the role of basal forebrain glutamatergic transmission in the regulation of cortical ACh release. First, ionotropic glutamate receptor activation is a necessary component for the increased cortical ACh release seen following exposure to the darkness/cereal stimulus. Second, the glutamate receptor subtypes differed in their ability to increase basal cortical ACh release in otherwise nonstimulated rats. Activation of AMPA/kainate receptors was sufficient to increase basal efflux whereas activation of NMDA receptors was not. Third, intrabasalis perfusion of NMDA potentiated the stimulated release produced by exposure to darkness/cereal. Finally, the procedure of repeatedly exposing the animals to the darkness/cereal stimulus resulted in long-lasting changes in the ability of NMDA to excite basal forebrain cholinergic neurons as the higher dose of NMDA was now able to increase basal release—an effect not seen in rats that were never exposed to the stimulus. The subsequent discussion will focus on three issues related to these findings: 1) the contributions of basal forebrain glutamate receptors to cortical ACh release under basal conditions, 2) the contributions of basal forebrain glutamate receptors to ACh release following the darkness/cereal stimulus, and 3) the particular interaction between NMDA receptor activity and the environmental stimulus.

### Intrabasalis forebrain glutamate receptors and basal cortical ACh release

The present results offer little evidence for a significant contribution of tonic ionotropic glutamatergic transmission to cortical ACh release under basal, nonstimulated conditions. Perfusion of the basal forebrain with the nonselective ionotropic glutamate receptor antagonist kynurenate only modestly reduced basal ACh efflux (Experiment 1). This result may have been due to a spurious increase in basal ACh efflux in vehicle-treated controls rather than a systematic effect of kynurenate on basal efflux. This conclusion is supported by a recent study in which kynurenate failed to decrease basal ACh in rats subsequently challenged with amphetamine (Fadel, unpublished observations). It is also not likely that the dose of kynurenate employed was insufficient to produce a decrease in basal ACh efflux. This same dose (1.0 mM) markedly suppressed the stimulated cortical ACh efflux seen follow-

ing the darkness/cereal manipulation. Even higher doses of intrabasalis kynurenate (5.0 or 10.0 mM) were reported not to depress basal cortical ACh release in anesthetized rats, yet again, these doses produced significant reductions in ACh efflux evoked by electrical stimulation of the pedunculopontine nucleus (Rasmusson et al., 1994). The conclusion that tonic glutamatergic transmission contributes little to basal cortical ACh release is further supported by the observation that intrabasalis perfusions of the more selective AMPA/KA antagonist DNQX were also without effect on ACh efflux. Again, doses of this antagonist (1.0 and 5.0 mM) that did not affect basal efflux eliminated kainate-induced increases in ACh efflux (Experiment 2). In contrast to these results, intrabasalis perfusions of the NMDA antagonist CPP (100 μM) have recently been reported to reduce basal cortical ACh efflux by approximately 50% (Giovannini et al., 1997). This result raises the interesting possibility that NMDA receptor activation contributes to basal ACh release but that the role of NMDA receptors in basal ACh release is not revealed by the effects of the selective antagonist kynurenate. The results of Giovannini et al. (1997) are also not consistent with the results in Experiment 3 in which intrabasalis perfusions of NMDA in nonstimulated animals were without effect of cortical ACh efflux. Given the results of Experiment 3, differences in the level of basal activity of cortical cholinergic inputs between animals in this experiment vs. those in Giovannini et al. (1997) could account for the inconsistent results regarding the role of NMDA receptor activation and basal cortical ACh efflux.

## Intrabasalis glutamate receptors and stimulated cortical ACh release

Several studies have demonstrated effects of intrabasalis administration of glutamate on cortical cholinergic transmission. Iontophoresis of glutamate into basal forebrain increases the firing rate of magnocellular, presumptive cholinergic, neurons (Lamour et al., 1986). Intrabasalis perfusions of glutamate increase cortical ACh efflux and result in a desynchronization of EEG (Kurosawa et al., 1989). Although caution must be exercised in comparing the effects of glutamate and its ability to simultaneously activate complex populations of ionotropic and metabotropic receptors on cortical cholinergic transmission in anesthetized rats with the present results, these findings, in conjunction with others (Giovannini et al., 1997), certainly indicate a potential for glutamatergic regulation of cortical ACh release. The results of Experiment 2 support this observation by revealing that selective stimulation of AMPA/KA receptors is a sufficient condition for the enhanced release of ACh in cortex.

## Interactions between basal forebrain NMDA receptors and environmental stimulation

Unlike the case with AMPA/KA receptors, stimulation of basal forebrain NMDA receptors did not affect basal cortical ACh efflux in rats tested under standard testing conditions (i.e., no discrete external stimulus). This difference between AMPA/KA and NMDA receptors may reflect the fact that only a small percentage of NMDA receptors are functional under basal conditions. NMDA receptor activation depends not just on agonist binding at the recognition site, but also on occupation of the "co-agonist" glycine site as well as concurrent membrane depolarization (to approximately −35 mV) to remove the voltage-dependent $Mg^{+2}$ blockade (Michaelis, 1998). Presentation of the darkness/cereal stimulus may provide the necessary shift in the balance of excitation/inhibition on basal forebrain cholinergic neurons, increasing the proportion of these cells which are depolarized enough to respond to NMDA. In this fashion, the perfusion of NMDA would then be able to potentiate the stimulating effects of darkness/cereal by increasing the number and duration of cholinergic corticopetal neurons that are firing.

It is unlikely that differences in the ability of intrabasalis glutamate agonists to increase cortical ACh efflux in these studies reflected simple differences in general arousal levels (as indicated by motoric output) between the two drugs. Intrabasalis kainate, for example, caused the greater increase in motor behaviors such as circling, rearing, and sniffing. Peak increases in cortical ACh efflux in this group, however, were far below those observed in the NMDA plus darkness/cereal group. This suggests that the large effect in the latter group was the result of a specific interaction between NMDA and the darkness/cereal stimulus (or its associated cues).

An unanticipated finding in Experiment 3 was the ability of NMDA (200 μM) to increase cortical ACh efflux in rats that had been repeatedly exposed to the darkness/cereal stimulus but before the presentation of the stimulus on the test day. This same dose of NMDA was completely without effect in the nonstimulated rats. We speculate that this potentiation of basal efflux in the repeatedly exposed rats reflects an enduring anticipatory activation of basal forebrain neurons resulting from reward-associated cues within the dialysis environment. This notion is supported by the well-established observation that basal forebrain cholinergic neurons respond not just to reward, but to stimuli that consistently precede reward (Richardson and De-Long, 1988). In this regard, it would be interesting to test animals that had been repeatedly exposed to darkness/cereal in a familiar, but nonreward-associated, space to determine whether presentation of palatable food represents a critical variable in the ability of NMDA to augment cortical ACh efflux.

Events that lead to such anticipatory activation may be further augmented by disinhibition of GABAergic inputs to basal forebrain by enhanced DA release in nucleus accumbens (see Moore et al., 1999). This speculation is supported by demonstration that cues related to repeatedly presented food reward increase DA efflux within accumbens (Richardson and Gratton, 1996) and that the increases in cortical ACh efflux that precede repetitive fixed-time daily feeding are antagonized by administration of positive allosteric modulators of GABA transmission such as the benzodiazepine agonist abecarnil (Ghiani et al., 1998). At the same time, the experience-induced shift in the sensitivity to NMDA seen in the present experiment might reflect an enduring shift in the strength of excitatory synaptic inputs to basal forebrain cholinergic neurons.

These experiments clearly demonstrate a capacity for glutamate receptor subtypes to stimulate or to potentiate increases in cortical ACh release. The specific sources of these glutamatergic inputs were not addressed in these microdialysis experiments. Glutamatergic inputs to the basal forebrain derive from cortical (primarily prefrontal) and amygdaloid sources (Gaykema et al., 1991; Zaborszky et al., 1997; Haring and Wang, 1986; Carnes et al., 1990) and the stimulatory effects of kainate in the present study may reveal a capacity for regulation by any or all of these afferent systems.

In conclusion, these microdialysis studies reveal that basal forebrain glutamate receptors can regulate cortical ACh release. The nature of this regulation, however, is complex and depends on the specific glutamate receptor subtype and the level of activation of the cholinergic corticopetal neurons. The well-established ability of repeated exposure to a darkness/cereal stimulus to enhance cortical ACh efflux (Fadel et al., 1996; Moore et al., 1992, 1993, 1995a) depends on basal forebrain glutamate receptor activity as intrabasalis perfusion of kynurenate severely attenuated this effect. Activation of AMPA/KA receptors with kainate, but not NMDA receptors with NMDA, stimulated basal release in nonstimulated animals. NMDA was able to potentiate the release seen following darkness/cereal and also became able to modulate basal release in these animals even before the presentation of the environmental stimulus. Future studies will be directed at defining the interactions between basal forebrain glutamate receptors and other modulating influences on cholinergic corticopetal neurons (i.e., GABAergic, dopaminergic) as well as the environmental/behavioral conditions that reveal these interactions.

## ACKNOWLEDGMENTS

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