BASAL FOREBRAIN CHOLINERGIC NEURONS: REGULATION BY DOPAMINE AND RESPONSES TO AROUSING STIMULI

By

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ABSTRACT

The regulation of forebrain cholinergic systems, specifically those neurons in the cholinergic basal nuclear complex (CBC) which project to the hippocampus and cortex are of great interest, given the involvement of acetylcholine (ACh) in cognitive function. To assess the activity of CBC neurons, *in vivo* microdialysis has been used in the present experiments to measure ACh release in the hippocampus and cortex of freely moving rats.

Dialysate concentrations of ACh in the hippocampus and cortex (and striatum) of freely moving rats were found to correlate positively and significantly with locomotor activity, a behavioural measure of arousal. Two arousing stimuli, injection of vehicle and onset of the rats' dark phase, increased locomotor activity and ACh release in all three brain regions, as did injection of the muscarinic antagonist scopolamine. These data suggest that forebrain cholinergic neurons are responsive to arousing stimuli and that ACh release in the cortex, hippocampus and striatum generally correlates with arousal.

The dopaminergic regulation of CBC neurons was examined by determining the extent to which dopamine (DA) receptor agonists and antagonists affect cortical and hippocampal ACh release. The indirect DA agonist *d*-amphetamine (AMPH) and the DA receptor agonist apomorphine increased ACh release in both the cortex and hippocampus as did the selective D_1 receptor agonist CY 208-243. D_2 receptor agonists (quinpirole and/or PHNO) had no effect on ACh release in the cortex and produced slight decreases in the hippocampus. In addition, the AMPH-induced increases in ACh release in both regions were attenuated more by the D_1 receptor antagonist SCH 23390 than by the D_2 antagonists haloperidol and/or raclopride, as was the apomorphine-induced release of ACh in the cortex. That DA mediates AMPH-induced increases in cortical ACh release was supported by the finding that prior selective lesions of

ascending dopaminergic but not noradrenergic systems attenuated this effect of AMPH. These results suggest that CBC neurons are regulated in an excitatory manner by DA acting primarily at D_1 receptors.

The extent to which ACh release in the cortex and hippocampus is related to the performance of a learned behavioural task was assessed in rats trained to anticipate and consume a palatable liquid diet. Hippocampal ACh release increased during the anticipatory and consummatory periods of the task, but the increase observed in rats trained with the liquid diet was not higher than the increases seen in rats trained with water or in naive rats. In contrast, cortical ACh release increased to a greater extent in rewarded rats than it did in the two control groups. This suggests that cholinergic activity in both the cortex and hippocampus is increased by a reward-independent aspect of the task, such as arousal or attention, while an additional reward-dependent component is seen with respect to cortical ACh release.

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For my mother, Barbara Day, for always expecting my best
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For Geert, an inspiring scientist and friend who is and will be greatly missed

I. INTRODUCTION

(A) THE ROLE OF ACETYLCHOLINE IN THE CENTRAL NERVOUS SYSTEM

As a result of Loewi's classic experiments in the early 1920's, acetylcholine (ACh) was the first chemical demonstrated to act as a neurotransmitter. These studies involved stimulating the vagus nerve of a frog, causing a decrease in heart rate, while perfusing the heart with a physiological solution. The solution recovered, when perfused through a denervated frog heart also decreased stimulation-induced heart rate, suggesting that a humoral factor was responsible for the effect of the nerve stimulation. This factor, termed "vagusstoff", was later identified as ACh. Eccles subsequently demonstrated in the 1950's that ACh could also act as a transmitter in the mammalian central nervous system (CNS). Dale had previously identified cholinergic transmission at the neuromuscular junction; based on this information and the famous Dale's principle, Eccles reasoned that ACh must also be the neurotransmitter responsible for the communication between the spinal motorneuron and the electrophysiologically identified Renshaw interneuron. These landmark studies were among the first of a great body of research on cholinergic systems which has helped to establish many of the basic principles of synaptic function and neurotransmission (see Karczmar, 1993).

Since these early studies, extensive research into the anatomy, chemistry, pharmacology and physiology of central cholinergic systems has yielded several well-accepted principles. For example, cholinergic neurons are now known to innervate much of the mammalian brain: nuclei in the brainstem send projections mainly to the thalamus and other diencephalic structures, neurons in the forebrain innervate the entire cortical mantle including the hippocampus and amygdala, and intrinsic cholinergic interneurons are found within the basal ganglia (Semba and Fibiger, 1989). Cholinergic neurons can be defined by the presence of the enzyme choline acetyltransferase (ChAT),

which catalyzes the synthesis of ACh from choline and acetylcoenzyme A (Tuček, 1984). Early investigations of cholinergic actions led to the discovery of two classes of receptors for ACh, nicotinic and muscarinic, which have more recently been subdivided based both on pharmacology and molecular biology (Ladinsky, 1993). The neurophysiological effects of ACh, mediated by nicotinic cation channel/receptor macromolecules and/or by G-protein coupled muscarinic receptors, are determined by the cellular location of the receptors and the specific second messenger systems and effectors to which they are coupled (Krnjević, 1993).

Given the complex anatomy of central cholinergic systems, the diversity of receptors which bind ACh, and the variety of ACh's electrophysiological effects, it is not suprising that ACh has complex and diverse roles in behaviour. Indeed, ACh neurotransmission has been reported to participate in a variety of behavioural functions including circadian rhythms (Rusak and Bina, 1990), antinociception (Iwamoto, 1989; Klamt and Prado, 1991), locomotion (Brudzynski *et al.*, 1991; Flicker and Geyer, 1982), regulation of the sleep/wake cycle (Baghdoyan *et al.*, 1993; Steriade *et al.*, 1991), stress (Gilad, 1987; Imperato *et al.*, 1991) and electrocortical arousal (Semba, 1991). Stimulated by the discovery that a consistent correlate of Alzheimer's dementia is a loss of cholinergic neurons in the basal forebrain (Coyle *et al.*, 1983), an intensively studied function of ACh has been its proposed role in learning and memory. Along with this correlational evidence of cholinergic loss in Alzheimer's dementia, studies have shown that administration of anticholinergic drugs to animals or humans or lesions of forebrain regions containing cholinergic cell bodies in animals cause cognitive impairments, which may be ameliorated by cholinergic agonists (Collerton, 1986; Hagan and Morris, 1988). These findings have supported a "cholinergic hypothesis of cognition", and have encouraged much research into possible cholinergic therapies for the treatment of cognitive deficits.

The excitement generated by this hypothesis has been tempered by the overall ineffectiveness of cholinergic therapies in treating dementia (Holttum and Gershon, 1992) and by criticisms of the above-mentioned supporting experimental evidence. For example, one major confound of the axon-sparing neurotoxic lesions used in animals to model Alzheimer's dementia is that non-cholinergic neurons in the vicinity of the target cholinergic neurons are lesioned as well (Arbogast and Kozlowski, 1988; Dunnett *et al.*, 1987; Everitt *et al.*, 1987). In fact, the extent to which excitotoxic lesions of the basal forebrain decrease cortical ChAT activity does not correlate with the severity of the resulting behavioural deficits in several learning and memory tasks (Dunnett *et al.*, 1987). Specifically, quisqualic acid lesions of the basal forebrain produce larger decreases in cortical ChAT, but less severe deficits in the acquisition and performance of several behavioural tasks, than do ibotenic acid lesions (Markowska *et al.*, 1990; Robbins *et al.*, 1989b). This suggests that the observed lesion-induced effects may be largely non-cholinergic in origin. Along with the lack of a selective cholinergic neurotoxin, several other shortcomings of the data supporting the cholinergic hypothesis of dementia have been outlined (Fibiger, 1991), including the idea that regionally specific effects of peripherally administered cholinergic drugs have not been considered.

Defining a cholinergic role in cognition is also complicated by the inherent difficulties in designing and interpreting the results of behavioural tasks. It is possible, for example, that the influence of ACh on cognition may be more accurately defined by the participation of forebrain cholinergic neurons in attentional processes rather than in learning and memory *per se*. Attention has various operational definitions but is generally assessed by a subject's ability to use sensory information to follow some rule or contingency; whereas a learning and memory task might assess the acquisition, retention or recall of this rule, an attention task assesses the subject's ability to follow the rule in situations where the initially salient sensory cue is unexpectedly switched to a new cue (attention reversal) or where the salient cue is presented among distractions (selective

attention). In support of a cholinergic involvement in attention, cholinergic agonists improve such measures of attention in Alzheimer's patients (Sahakian *et al.*, 1989; 1993) and the cholinergic receptor antagonist, scopolamine, disrupts attention in normal human subjects (Dunne and Hartley, 1985; Wesnes and Warburton, 1983). Additionally, excitotoxic lesions or pharmacological inhibition of the basal forebrain in animals causes impairments in attentional tasks (Olton *et al.*, 1988; Pang *et al.*, 1993; Robbins *et al.*, 1989a; Voytko *et al.*, 1994) which, in some cases, have been ameliorated by cholinergic agonists or tissue grafts (Muir *et al.*, 1992a; 1992b; 1994). It is important to note, however, that these experiments suffer from the same limitations as those described above (for example, lack of a selective cholinergic neurotoxin), and that contradictory data have also been reported (Roberts *et al.*, 1992). It should also be noted that these results do not exclude a role for basal forebrain cholinergic neurons in learning and memory. It has recently been suggested, for example, that excitotoxic lesions of basal forebrain regions more rostral than those which cause attention deficits may impair memory (Muir *et al.*, 1993).

Learning, memory and attention are complex cognitive constructs, operationally defined using behavioural measures. In contrast, electroencephalographic (EEG) arousal is an electrophysiological measure, defined by low voltage fast activity (LVFA), or desynchrony, in the cortex and rhythmic slow activity (RSA), or theta rhythym, in the hippocampus. The importance of ACh in the generation of these averaged electrical patterns is well supported and has been reviewed (Buzsáki and Gage, 1989; Riekkinen *et al.*, 1991a; Semba, 1991; Smythe *et al.*, 1992; Vanderwolf, 1988). Briefly, the activity of presumed cholinergic neurons in the basal forebrain is increased during EEG arousal (Détári and Vanderwolf, 1987; Sweeney *et al.*, 1992); stimulation of cholinergic cell body regions induces LVFA (Metherate *et al.*, 1992); RSA can be induced or blocked by administration of cholinergic agonists or antagonists, respectively, into the hippocampus (Golebiewski *et al.*, 1992; Rowntree and Bland, 1986) or the basal forebrain (Monmaur

et al., 1993); cortical ACh release is increased during LVFA (Casamenti *et al.*, 1986; Celesia and Jasper, 1966; Kanai and Szerb, 1965); and cholinergic antagonists or lesions of cholinergic cell body regions disrupt EEG arousal (Buzsáki *et al.*, 1988; Ray and Jackson, 1991; Riekkinen *et al.*, 1992) which in some cases can be ameliorated by cholinergic agonists (Riekkinen *et al.*, 1991a; Vanderwolf *et al.*, 1993).

Although the critical significance of ACh in EEG arousal is well established, the mechanisms by which ACh and other neurotransmitters interact to create the electrophysiological phenomena underlying LVFA and RSA are complex and still being elucidated (Foote and Morrison, 1987; Smythe *et al.*, 1992, Steriade *et al.*, 1993; Vanderwolf, 1988). In addition, the behavioural significance of EEG arousal has not been definitively determined. It should be noted, for example, that LVFA and RSA occur during rapid eye movement (REM) sleep. In the waking state, however, EEG arousal occurs during voluntary movement in laboratory animals (Vanderwolf, 1988) and during periods of facilitated sensory processing (Livingstone and Hubel, 1981; Simon and Emmons, 1956). It is thus often speculated, but not universally accepted (Vanderwolf, 1988), that EEG arousal may be a physiological representation of attentiveness, and may be required for cognitive functioning. This theory is supported by correlational evidence including reports that the degree of disruption of EEG patterns correlates with the severity of cognitive deficits in Alzheimer patients (Penttilä *et al.*, 1985) and that cognition enhancers activate the EEG (Delacour *et al.*, 1990a). The fact that LVFA and RSA also occur during REM sleep may not dispute the theory that aroused EEG patterns are required for cognitive functioning, given that REM sleep may be involved in information processing (Dujardin *et al.*, 1990).

Consistent with the speculation that increased ACh release occuring during EEG arousal contributes to increased attentiveness, cholinergic mechanisms are involved in facilitating the activity of hippocampal (Markram and Segal, 1990) and cortical neurons responding to afferent neural transmission. Single or multiple unit responses of cortical

neurons to visual, auditory or somatic stimulation, or to stimulation of the auditory thalamus, are enhanced by iontophoretic application of ACh and/or by stimulation of the basal forebrain (Donoghue and Carroll, 1987; Hars *et al.*, 1993; Metherate and Ashe, 1993; Metherate *et al.*, 1987; Sillito and Kemp, 1983; Tremblay *et al.*, 1990; Webster *et al.*, 1991). In some cases, the facilitory effects of basal forebrain stimulation have been blocked by intracortical iontophoretic or systemic administration of the muscarinic receptor antagonist atropine (Hars *et al.*, 1993; Metherate and Ashe, 1993; Tremblay *et al.*, 1990). Electrical stimulation of the basal forebrain may approximate an increase in the firing of basal forebrain neurons, such as that which occurs in monkeys presented with stimuli that precede, or have been previously paired with reinforcement (Richardson and DeLong, 1990; Wilson and Rolls, 1990) or in rabbits presented with a conditioned stimulus which predicts a mildly aversive stimulus (Whalen *et al.*, 1994). Accordingly, the increased cortical ACh release presumably accompanying this increased firing may facilitate cortical neuronal responses to other, co-incident afferent signals, thereby providing a mechanism by which the relevance of sensory stimuli could be neurally encoded. Cholinergic facilitation of neuronal responsiveness may also participate in sensory cortex plasticity (Delacour *et al.*, 1990b), cellular associative conditioning (Pirch *et al.*, 1992; Rigdon and Pirch, 1986), long-term potentiation in both the cortex and hippocampus (Blitzer *et al.*, 1990; Hunter *et al.*, 1994; Lin and Phillis, 1991) and afferent stimulation-induced protein synthesis in the hippocampus (Feig and Lipton, 1993), all of which have been proposed as cellular models or correlates of learning and memory.

Considered together, the research outlined above points to the importance of forebrain cholinergic systems in determining an animal's perception of its environment as to which aspects are deemed significant, attended to, and thus perhaps remembered. Although terms such as learning, memory, attention and arousal may be insufficiently precise to define the roles of ACh in the CNS, they do succeed in suggesting a role for

this neurotransmitter in cognitive function. To complement research aimed at determining the functional significance of forebrain cholinergic systems, basic characterizations of these neurons are required, including their response to environmental stimuli and their regulation by endogenous neurotransmitters. Given the anatomy and proposed functions of cholinergic systems, such research should ideally include measures of regionally specific cholinergic activity in awake, behaving animals.

(B) *IN VIVO* MICRODIALYSIS AND ASSAY OF ACH

In vivo microdialysis allows the measurement of interstitial concentrations of neurotransmitters in discrete brain regions of awake, behaving animals (Benveniste and Hüttemeier, 1990; Ungerstedt, 1984; Westerink *et al.*, 1987) and is presently the best available technique for estimating ACh release. Microdialysis exploits the ability of small molecules to diffuse down a concentration gradient from the brain into a solution approximating brain interstitial fluid perfused through a semi-permeable, tubular dialysis membrane. A neurochemical which is permeable to the dialysis membrane, and for which there is a sensitive assay, can then be measured in the collected dialysate. Similarly, using reverse dialysis, drugs included in the perfusion solution can be applied by diffusion into the discrete sampling area of the dialysis probe, and this can be accomplished concurrently with neurochemical measurement. An obvious advantage of microdialysis over *ex vivo* techniques is the ability to monitor neurochemicals frequently (several times an hour) and for many hours in each animal. Compared to earlier *in vivo* sampling methods, such as push-pull perfusion (Philippu, 1984) and the cortical-cup technique (Moroni and Pepeu, 1984), microdialysis produces less tissue damage and perturbation, can be performed in most brain regions, and is amenable to use in awake, freely-moving animals. Microdialysis also has an advantage over another current *in vivo*

neurochemical sampling technique, *in vivo* electrochemistry (Kawagoe *et al.*, 1993), which can only be used to detect electroactive neurochemicals.

Despite its many advantages, *in vivo* microdialysis has several limitations which must be considered. First, the concentrations of neurotransmitters measured using microdialysis do not represent absolute synaptic concentrations. Factoring into the calculations the *in vitro* recovery of the dialysis probe succeeds in estimating the concentration of molecules reaching the dialysis probe, but neglects the influence of important *in vivo* factors (Amberg and Lindefors, 1989; Benveniste *et al.*, 1989; Justice, 1993) such as metabolism, active uptake and tortuosity, which is a descriptor of the impedance of the interstitial pathway through which the transmitter diffuses between its release site and the probe. Because the absolute synaptic concentration of transmitter molecules can not yet be accurately determined, most microdialysis data are interpreted with regard to relative changes in interstitial concentrations of transmitter arising, presumably, from synaptic overflow.

The possibile origin of the recovered transmitter from activity-independent release from neurons or from non-neuronal sources is the second limitation of microdialysis that must be considered. To assess the degree to which diaysate concentrations of a neurochemical are dependent on activity-dependent release, the sodium channel blocker tetrodoxin (TTX) can be added to the perfusion solution, effectively blocking the conduction of action potentials in the vicinity of the dialysis probe. In optimal microdialysis conditions, TTX treatment reduces the concentrations of several neurotransmitters by at least 80%, and often to below detectable levels (Damsma *et al.*, 1987b;1987c; Santiago and Westerink, 1990; van Veldhuizen *et al.*, 1990). Removal of calcium ions from the perfusion solution, or their replacement with competitive bivalent cations, can also be used to estimate the proportion of dialysate transmitters originating from calcium-dependent vesicular release (Benveniste and Hüttemeier, 1990), although

non-vesicular, calcium-independent release may still be neuronal in origin and activity related (Levi and Raiteri, 1993).

It has been demonstrated that the tissue trauma resulting from probe implantation affects interstitial concentrations of neurochemicals (de Boer *et al.*, 1992; Reiriz *et al.*, 1989); the amount of time after implantation required to optimize recovery of neurochemicals related to activity-dependent release, usually one to two days, depends in part upon the type of dialysis probe used (Santiago and Westerink, 1990). In the case of neurochemicals such as glutamate and adenosine, which have constitutive roles in cellular functioning as well as proposed roles as neurotransmitters, those molecules originating from neurotransmission-related release may only represent a fraction of the total amount recovered. Thus, for example, basal microdialysate concentrations of glutamate are unaffected or only partially reduced by TTX infusion (Girault *et al.*, 1986; Moghaddam, 1993; Westerink *et al.*, 1987).

A third important consideration to be made concerning microdialysis is whether basal, unstimulated neurotransmitter release is being measured. Dialysate concentrations of neurotransmitters have been shown to be dependent on the ionic composition of the perfusate (Moghaddam and Bunney, 1989; Osborne *et al.*, 1991). Perfusate solutions containing higher concentrations of calcium or potassium than those found in the interstitial space surrounding the probe would be expected to artificially stimulate transmitter release by affecting the respective ion gradients and currents across the neuronal membrane. Indeed, it has been shown that perfusion solutions differing in calcium concentration can yield qualitatively different results in ACh microdialysis experiments (de Boer *et al.*, 1990a). To ensure that basal release is being assessed, the ionic composition of the perfusate should closely approximate that in the interstitial space (Hansen, 1985).

A final characteristic of microdialysis that must be considered is the basic functional principal of dialysis itself: that any molecule or ion which is permeable to

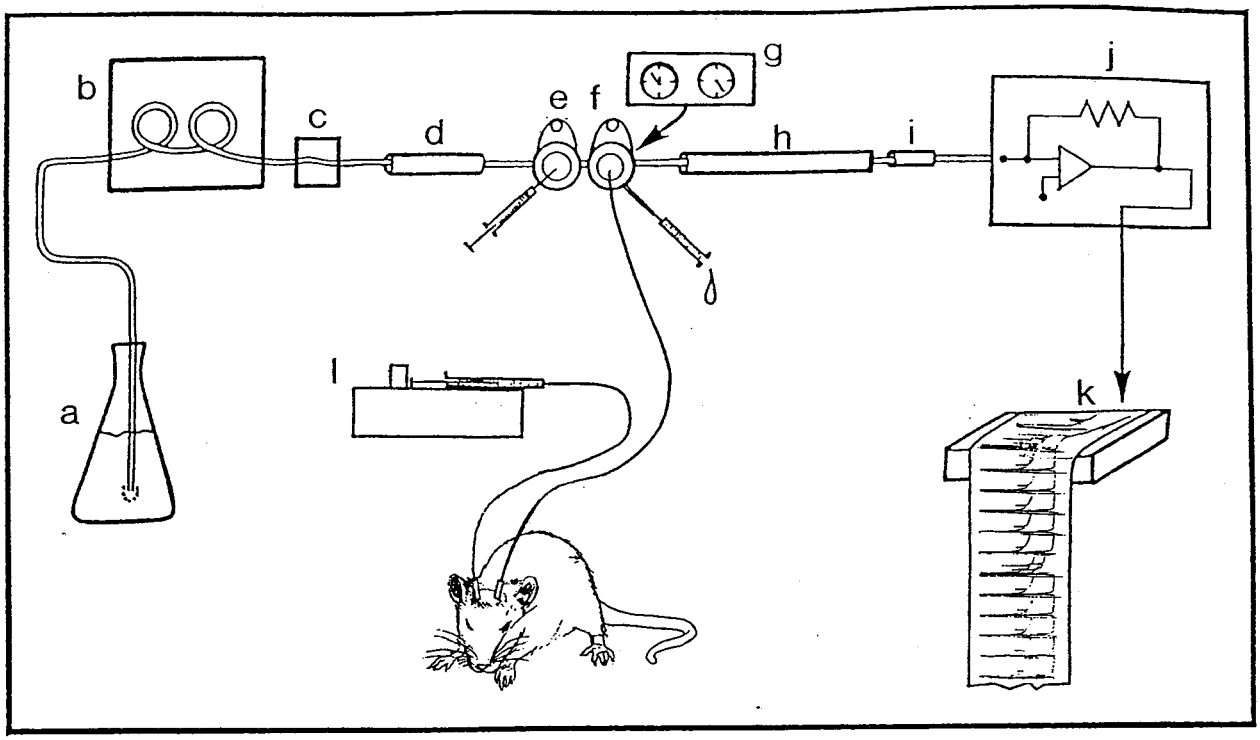
the dialysis membrane and which is not in equal concentrations in the brain and in the perfusion solution will cross the membrane, flowing down its concentration gradient. Although this allows the recovery and analysis of neurochemicals, it also disturbs the local chemical microenvironment by removing not only the molecular species of interest but also many others. The brain tissue surrounding the probe for up to 1mm is drained by this neurochemical "sink" (Benveniste, 1989; Blaha, 1991) and may be influenced by such factors as rate of perfusion, perfusate composition, and probe geometry. It must therefore be recognized that microdialysis does not assess neurotransmitter release in entirely normal physiological conditions, but rather within a zone of artificial equilibrium between the tissue and the dialysis probe, where many dynamic neuronal processes are presumably affected. The recently developed technique of quantitative dialysis (Justice, 1993) attempts to deal with this issue, at least with respect to the species of interest, by including this neurochemical in the perfusate at a concentration where no net flow occurs across the membrane. While this modification is prohibitively labour-intensive for most dialysis applications, it is informative about the dialysis technique itself.

A special consideration must be made in the case of microdialysis for recovery of ACh. Because the degradation of released ACh by acetylcholinesterase (AChE) activity is extremely efficient, an AChE inhibitor is usually included in the perfusion solution. The reversible, non-lipophilic, quaternary AChE inhibitor neostigmine has been argued to be best suited for this purpose (Damsma and Westerink, 1991). Depending on the sensitivity of the specific assay, this adaptation is necessary in most cases to allow the recovery of reliably detectable amounts of ACh. High-maintenance assay procedures or long sampling intervals have been used to carry out dialysis experiments in the absence of an AChE inhibitor (Damsma *et al.*, 1987c; de Boer *et al.*, 1990b; Messamore *et al.*, 1993), although it is usually reported that release is not detectable in all animals tested. To increase the number of successful experiments, inclusion of low concentrations of an

AChE inhibitor is generally considered an acceptable compromise of the dialysis technique. Indeed, presently it is not known to what extent the presence of AChE inhibition might actually ameliorate the above-mentioned dialysis "sink" effect, at least with respect to the local depletion of ACh surrounding the membrane.

Although the above-mentioned issues must be considered in assessing microdialysis as an *in vivo* neurochemical sampling technique, the question of quantitative assays for ACh need only address two points: sensitivity and specificity. Several assays for ACh have been coupled with *in vivo* monitoring techniques, including bioassays (Celesia and Jasper, 1966; Kanai and Szerb, 1965), gas chromatography/mass spectrometry (Marien and Richard, 1990), a radioenzymatic assay (Consolo *et al.*, 1987) and a radioimmunoassay (Kawashima *et al.*, 1991). The highest sensitivities routinely reported, however, have been from chromatographic methods coupled to electrochemical detection (ECD; Damsma *et al.*, 1987a). ECD of the electrically inactive ACh molecule is achieved by post-chromatographic enzymatic conversion of ACh, *via* an acetylcholinesterase, to choline and acetic acid, and the subsequent conversion of choline, *via* choline oxidase, to betaine and hydrogen peroxide (Fig. 1). The hydrogen peroxide, stoichiometrically produced from ACh, is detected by its oxidation at a platinum electrode. The chromatographic separation and subsequent enzymatic processing of the ACh fulfills the requirement of specificity in the assay.

Given the above-mentioned considerations, the experiments reported here use *in vivo* microdialysis coupled to on-line assay of ACh by high performance liquid chromatography with ECD (HPLC-ECD; Damsma and Westerink, 1991) to assess neuronal overflow of ACh in forebrain regions of awake, freely moving rats (Fig. 1). Experiments were conducted on the second day after implantation of a transverse microdialysis probe and 100 nM neostigmine was included in the perfusate. Microdialysate concentrations of ACh using this methodology have previously been demonstrated to be calcium-dependent and TTX-sensitive (Damsma *et al.*, 1987c; 1988).



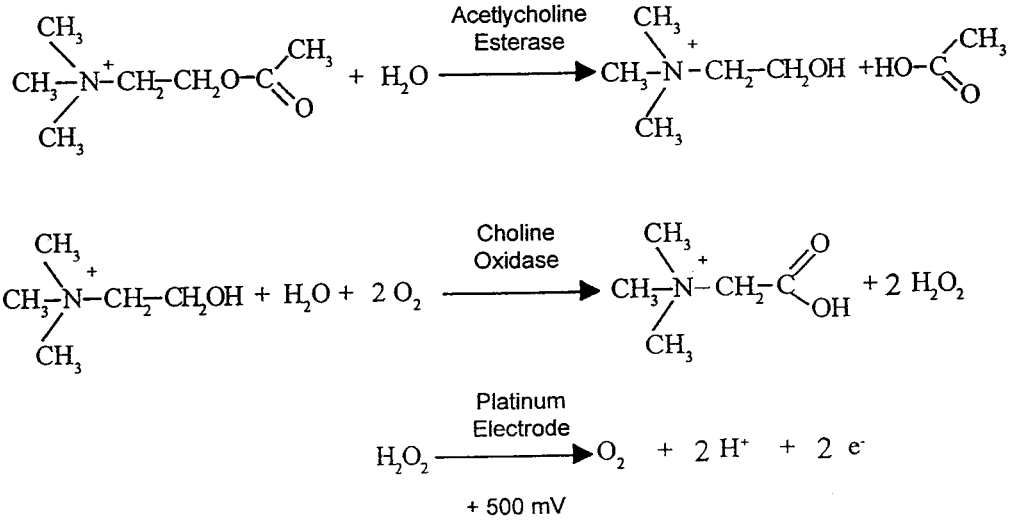


Fig. 1 Upper panel: Schematic of *in vivo* microdialysis with on-line assay for acetylcholine (ACh). a) Mobile phase reservoir, b) HPLC pump, c) pulse dampener, d) guard column, e) standard loop and valve, f) sample loop and valve, g) timer, h) analytical column, i) immobilized enzyme reactor, j) electrochemical detector including working and reference electrodes and potentiostat, k) chart recorder and l) perfusate-syringe driver. An average time delay of 20 min occurs between the neurochemical event in the rat's brain and its representation on the chart recorder. See Chapter II, Materials and Methods section for details.
Lower panel: Steps in the enzymatic conversion and electrochemical detection of ACh, occuring in the enzyme reactor and on the working electrode (i and j above).

II. ACETYLCHOLINE RELEASE IN THE RAT HIPPOCAMPUS, CORTEX AND STRIATUM CORRELATES WITH BEHAVIOURAL AROUSAL

(A) INTRODUCTION

ACh has been implicated in a variety of behavioural functions including learning and memory (Collerton, 1986; Hagan and Morris, 1988). Although the strength of the data supporting the cholinergic hypothesis of dementia has been questioned (Fibiger, 1991), forebrain ACh may participate in cognitive functions other than learning and memory *per se*, such as attention (Muir *et al.*, 1994; Sahakian *et al.*, 1993; Voytko *et al.*, 1994) or arousal (Semba, 1991). Arousal can be operationally defined in laboratory animals either electroencephalographically or behaviourally. Central cholinergic neurotransmission has been associated with both EEG and behavioural measures of arousal. Using cortical cup and push/pull perfusion techniques, increased concentrations of ACh have been recovered during spontaneous or stimulated EEG desynchronization in cats (Celesia and Jasper, 1966; Kanai and Szerb, 1965; Phillis and Chong, 1965; Szerb, 1967) and rats (Casamenti *et al.*, 1986), during increased behavioural activity in rabbits (Collier and Mitchell, 1967), and during sensory stimulation or treadmill-running in rats (Dudar *et al.*, 1979). More recently, significant correlations have been reported between cortical, hippocampal and striatal dialysate ACh concentrations (or whole brain tissue concentrations) and motor activity counts after anticholinergic treatment (Frances and Jacob, 1971; Toide, 1989; Watanabe and Shimizu, 1989).

Microdialysate concentrations of ACh from awake, freely-moving rats in drug-free conditions have been demonstrated to vary considerably (Damsma *et al.*, 1987b), and preliminary studies in this laboratory suggested that these variations may be due to changes in animals' activity levels. The experiments reported here were undertaken to further examine the possible relationship between ACh release and arousal, as measured

by locomotor activity. Photocell beam interruptions and *in vivo* microdialysate concentrations of ACh and choline (Ch) in the dorsal hippocampus, frontal cortex or dorsal striatum of rats were measured simultaneously under three conditions: 1) after an injection of vehicle; 2) after administration of the muscarinic receptor antagonist, scopolamine; and 3) before and after the beginning of the rats' night cycle. The two non-pharmacological stimuli are associated with increased locomotion, while muscarinic receptor antagonists also increase locomotion and are known to increase ACh release in the cortex, hippocampus, and striatum *via* the blockade of an inhibitory feedback mechanism (Aquilonius *et al.*, 1972; Dolezal and Wecker, 1990; Lefresne *et al.*, 1978; Nordstrom and Bartfai, 1980; Rospars *et al.*, 1977; Szerb *et al.*, 1977).

(B) MATERIALS AND METHODS

Experimental protocol and drugs

Experiments were performed on male Wistar rats (250-330g) two days after the implantation of a microdialysis probe. Following surgery, rats were housed individually in Plexiglas cages (35x35x25 cm) and maintained on a 12:12 h light:dark schedule with food and water available *ad libitum*. During the light phase of the rats' daily cycle, subjects were first injected with water (1 ml/kg, s.c.), and then 3 h later with the muscarinic antagonist, scopolamine hydrobromide (0.4 mg/kg, Sigma). Experiments continued into the rats' usual dark phase (when the room lights were turned off) approximately 4 h later. During each of these conditions, locomotor activity and hippocampal, cortical or striatal interstitial concentrations of ACh and Ch were measured simultaneously.

Surgery and microdialysis

Trans-cerebral microdialysis sampling of ACh was performed according to the methodology of Damsma *et al.* (1987b) which has been reviewed (Damsma and Westerink, 1991). Rats were stereotaxically implanted with a transverse dialysis probe (Fig. 2; Damsma *et al.*, 1987b; Imperato and Di Chiara, 1984) under pentobarbital anesthesia (50-60 mg/kg, i.p.). The probe was placed in one of three sites according to the atlas of Paxinos and Watson (1986), measured from bregma in mm: striatum A:+1.7, V:-4.75; hippocampus A:-4.3, V:-3.3; frontal cortex A:+2.7, V:-2.5. The probes were made of saponified cellulose ester dialysis fibre (ID = 0.22 mm, OD = 0.27 mm, molecular weight cut off > 10 000 Dalton; Cordis Dow Medical), having an active surface length of 6.4 mm, 6.8 mm, or 10.9 mm for the striatum, hippocampus, or frontal cortex, respectively. On completion of each experiment, the probe location was verified using standard histological procedures.

During microdialysis experiments the dialysis fibre was perfused at 5 *u*l/min, controlled by a syringe pump (Carnegie Medicin). The syringe was connected to the probe inlet by polyethylene tubing (800x0.28 mm); the probe outlet was connected to the sample loop (100 *u*l) of the analytical system by fused silica tubing (800x0.1 mm). The sample valve was controlled by an adjustable timer (Valco), and samples (50 *u*l) were collected and injected at ten minute intervals.

The composition of the perfusion solution was selected to approximate the ionic composition of the interstitial fluid in the brain (Hansen, 1985) and contained NaCl (125 mM), KCl (3 mM), $CaCl_2$ (1.3 mM), $MgCl_2$ (1.0 mM), $NaHCO_3$ (23 mM) in aqueous phosphate buffer (pH 7.3). To recover detectable dialysate concentrations of ACh, a reversible acetylcholinesterase (AChE) inhibitor (neostigmine bromide, 0.1 *u*M; Sigma) was included in the perfusion solution. Thirty minutes of perfusion preceded sample collection to allow perfusate concentrations of ACh and Ch to equilibrate with those in the brain.

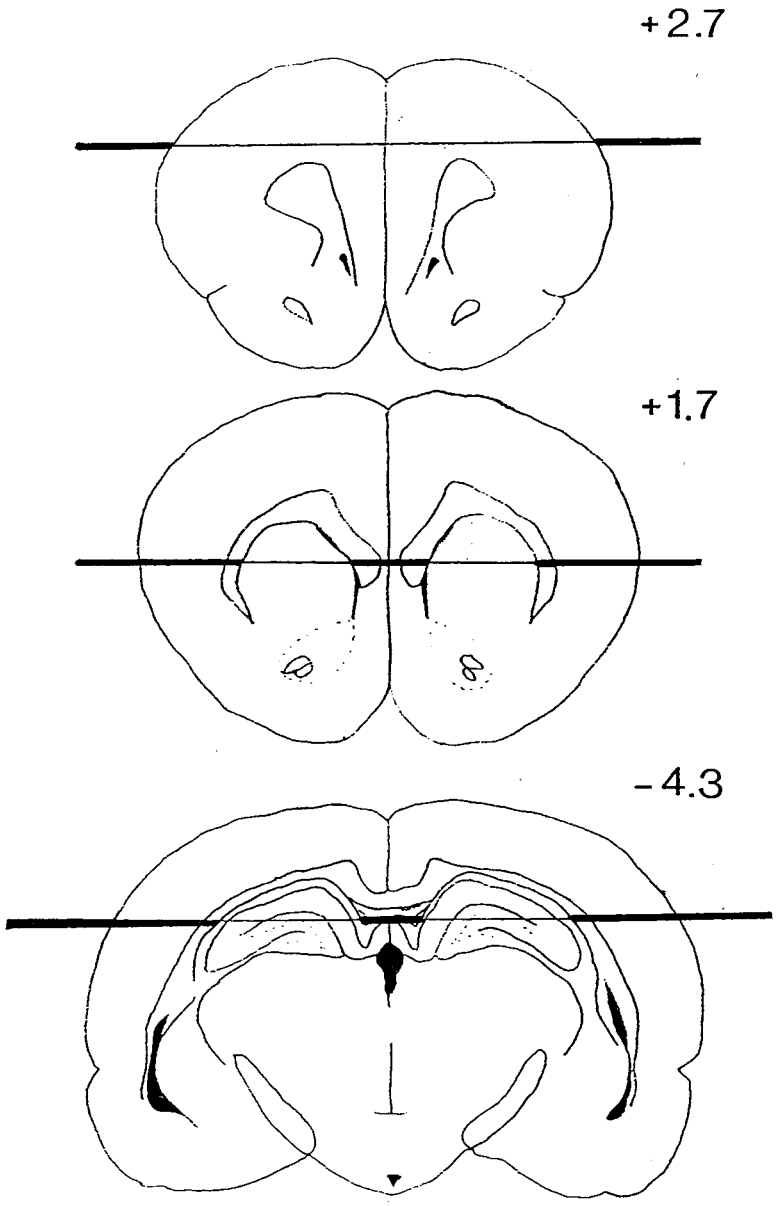


Figure 2. Dialysis probe placements in the frontal cortex, dorsal striatum and dorsal hippocampus. Measurements are from Bregma, in mm. Thinner areas of the probe represent those areas of the fibre open to dialysis, whereas the thicker areas are blocked by glue.

Assay of ACh

ACh was assayed by HPLC-ECD in conjunction with an enzyme reactor (Damsma *et al.*, 1987a; Fig. 1). ACh and Ch were separated on a reverse phase column (75x2.1 mm) pretreated with lauryl sulphate. The eluent from this analytical column then passed through an enzyme reactor (10x2.1 mm) containing acetylcholinesterase (EC 3.1.1.7; Sigma, type VI-S) and choline oxidase (1.1.3.17; Sigma), covalently bound to glutaraldehyde-activated Lichrosorb NH_2 (10 *u*m; Merck). The separated ACh and Ch reacted to give a stoichiometric yield of hydrogen peroxide, which was electrochemically detected at a platinum electrode at a potential of +500 mV versus an Ag/AgCl reference electrode (BAS-LC4B). The mobile phase, 0.2 M aqueous potassium phosphate buffer pH 8.0, was delivered by a pump (LKB-2150) at 0.4 ml/min. The detection limit of the assay was approximately 50 fmol/injection. The time required to complete a chromatogram was 4-5 min. Standards of ACh/Ch were injected hourly.

Motor activity

During the microdialysis experiments, a Digiscan Animal Activity Monitor (model RXYZCM(16); Omnitech Electronics, Inc.) was used to measure locomotor activity in 10 min blocks corresponding to the 10 min dialysate samples. Successive interruptions of the same photocell beam were categorized by the monitor as "stereotypy", and these stereotypy counts were subtracted from the total horizontal counts to yield a measure of ambulation.

Statistical analyses

Biochemical data were calculated as a percent of baseline concentrations, 100% baseline being defined as the average of the last three pre-scopolamine values. A univariate analysis of variance (ANOVA) with repeated measures was used to compare the effects of scopolamine and vehicle on ACh and Ch output. ANOVAs were also

conducted to evaluate the effects of the vehicle injection and exposure to dark on ACh and Ch. These analyses included the three samples prior to and the three samples after the initiation of these two treatments. Comparisons of the biochemical responses to the three experimental variables between brain regions were also made using ANOVAs. Greenhouse-Geisser adjustments of the degrees of freedom were made to account for the use of time as a repeated measure. All reported values refer to the interaction effect of time with experimental treatment. Pearson's correlation coefficients between ACh concentrations (in fmol/min) and motor counts were determined for the three treatment conditions in each individual rat. Group correlations were carried out as well, in which the data of all animals having probes in the same brain region were combined for analysis. In this case, the biochemical measures were expressed as percentage of baseline. Because motor counts were not normally distributed, for all the correlation analyses these values were normalized using logarithmic transformation.

(C) RESULTS

The average baseline outputs of ACh and Ch in the three brain regions are shown in Table 1 and have been corrected for the differences in probe surface length.

The neurochemical and motor effects of vehicle and scopolamine injections, and exposure to dark, are shown in Figs. 3, 4 and 5 for the striatum, hippocampus and cortex, respectively. Vehicle injections transiently increased extracellular ACh by 54% in the hippocampus ($p<0.05$) and by 161% in the frontal cortex (n.s.), while no change was evident in the striatum. No significant effect of this treatment on dialysate concentrations of Ch was observed in any brain region. The responses of ACh and Ch in the three brain regions to vehicle treatment were not significantly different. Injection of vehicle caused short lasting increases in motor activity.

Table 1. Probe characteristics and baseline output of ACh and Ch in the striatum, hippocampus and frontal cortex of rats

Brain Region	Baseline Dialysate Output (fmol/min/mm) ACh	Ch	Probe Surface Length (mm)	*In vitro* Probe Recovery (%)
Striatum	9.04 ± 0.67	588 ± 130	6.4	21.4 ± 6.2
Hippocampus	2.10 ± 0.48	368 ± 82	6.8	22.5 ± 5.4
Frontal cortex	2.30 ± 0.56	294 ± 82	10.9	33.3 ± 7.6

Dialysate outputs are expressed as the mean (± S.E.M.) of the last 3 pre-scopolamine values from 4-6 rats. Outputs are corrected for the active surface length of the probes, as shown. Neostigmine (100 nM) was included in the perfusion solution.

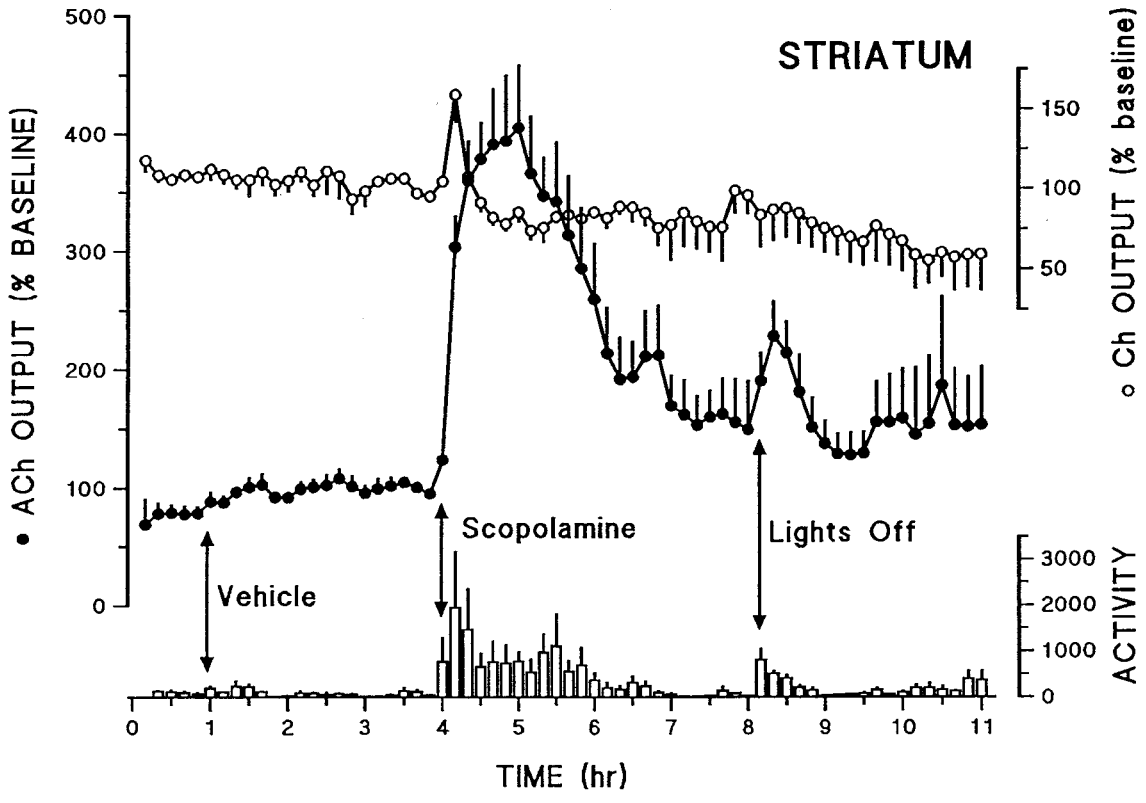


Figure 3. Striatal dialysate values of ACh (closed circles) and Ch (open circles) after injections of vehicle and scopolamine and exposure to dark. Activity counts (open bars) were measured concurrently. Vehicle (1 ml/kg) and scopolamine (0.4 mg/kg) were injected subcutaneously, and "lights off" occured at the appropriate time in the rats' daily cycle. Data points represent group means (n=4-6) ± S.E.M.

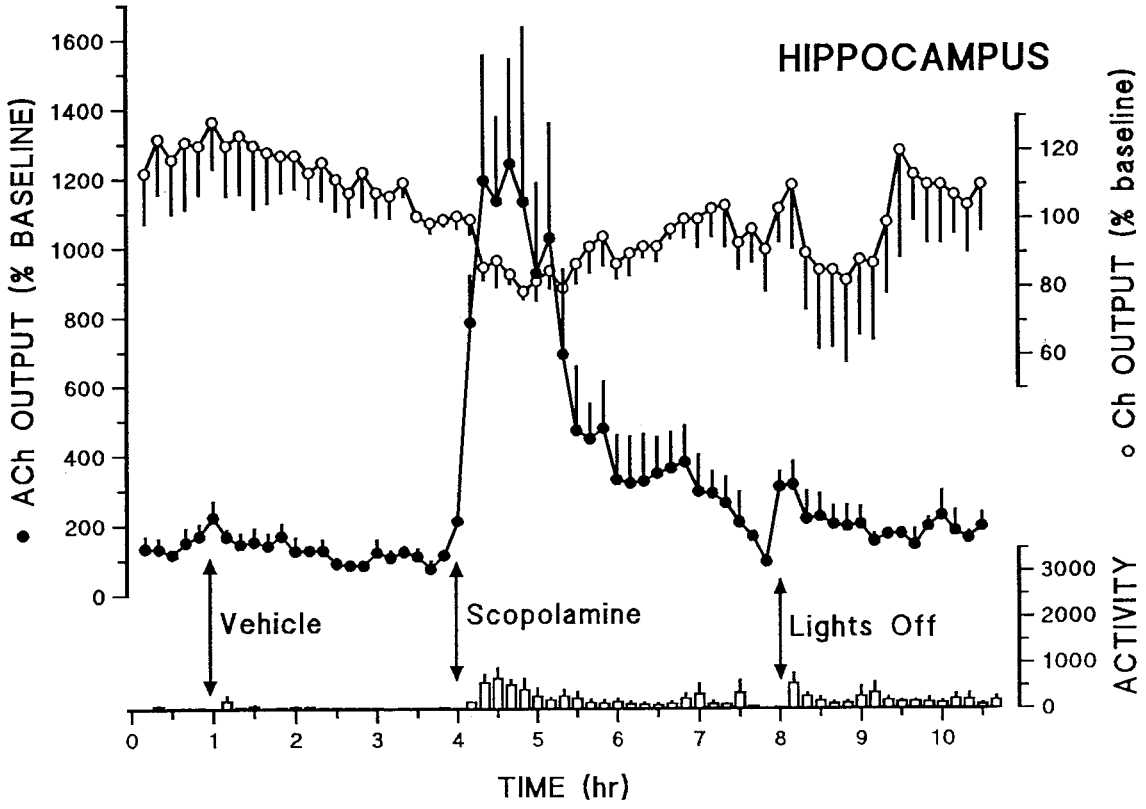


Figure 4. Hippocampal dialysate values of ACh (closed circles) and Ch (open circles) after injections of vehicle and scopolamine and exposure to dark. Activity counts (open bars) were measured concurrently. Vehicle (1 ml/kg) and scopolamine (0.4 mg/kg) were injected subcutaneously, and "lights off" occured at the appropriate time in the rats' daily cycle. Data points represent group means (n=4) ± S.E.M.

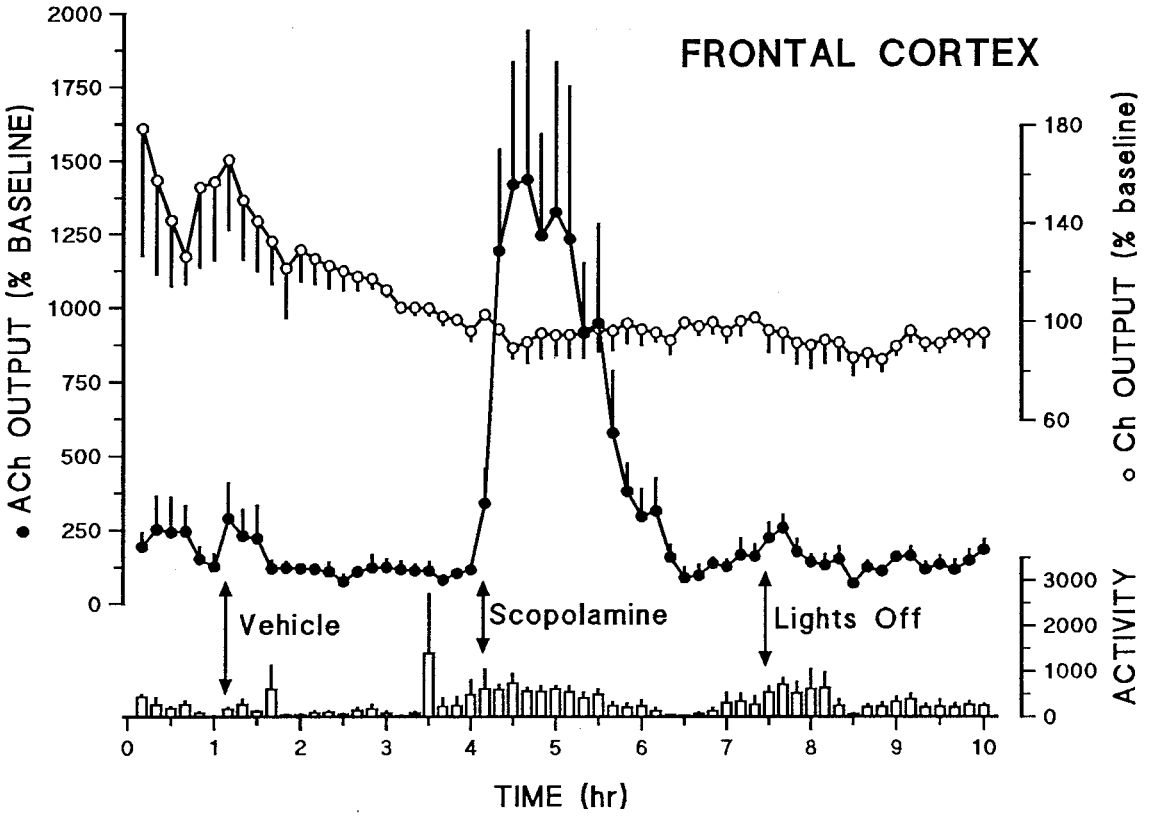


Figure 5. Frontal dialysate values of ACh (closed circles) and Ch (open circles) after injections of vehicle and scopolamine and exposure to dark. Activity counts (open bars) were measured concurrently. Vehicle (1 ml/kg) and scopolamine (0.4 mg/kg) were injected subcutaneously, and "lights off" occured at the appropriate time in the rats' daily cycle. Data points represent group means (n=4-5) ± S.E.M.

Scopolamine (0.4 mg/kg, s.c.) caused large increases in interstitial ACh and in motor activity. Peak ACh increases of 400, 1200 and 1400% in the striatum, hippocampus and cortex, respectively, occurred within 20 min and persisted for approximately 1 h. Compared to vehicle values, scopolamine had a significant effect on ACh output (striatum $p<0.001$, hippocampus and cortex $p<0.05$). The drug significantly affected Ch only in the striatum ($p<0.01$) where there was an increase in the second post-injection sample (159% of baseline). The effect of scopolamine on ACh was significantly smaller in the striatum than in either the cortex or hippocampus ($p<0.05$). The drug responses in the hippocampus and cortex did not differ significantly from each other. Motor activity was greatly increased by scopolamine with approximately the same time course as was observed for the increase in ACh.

At the onset of the dark phase of the rats' day-night cycle, motor activity increases coincided with ACh increases of 58% in the striatum, 169% in the hippocampus, and 77% in the cortex. In the striatum, ACh showed a significant illumination by time interaction effect ($p<0.05$); the increases in the other areas failed to reach significance. The three brain regions did not differ significantly with respect to the ACh nor Ch response to dark.

To illustrate the nature of the relationship between behavioural activity and ACh measured in either the striatum, hippocampus or cortex, Fig. 6 shows the motor and dialysate profiles of three individual animals. Most of the animals tested exhibited significant correlations between locomotor activity and ACh release under all three experimental conditions. After vehicle injection, significant correlations ($p<0.05$) were found in 2 of 4 animals with striatal probes ($r=0.572-0.643$), 3 of 4 animals with hippocampal probes ($r=0.611-0.808$) and in 3 of 5 animals with frontal cortex probes ($r=0.665-0.704$). Scopolamine treatment yielded significant values in all 6 animals with striatal probes ($r=0.532-0.867$), in all 4 with hippocampal probes ($r=0.517-0.736$) and in 4 of 5 animals with frontal cortex probes ($r=0.741-0.877$). During lights-off, motor counts

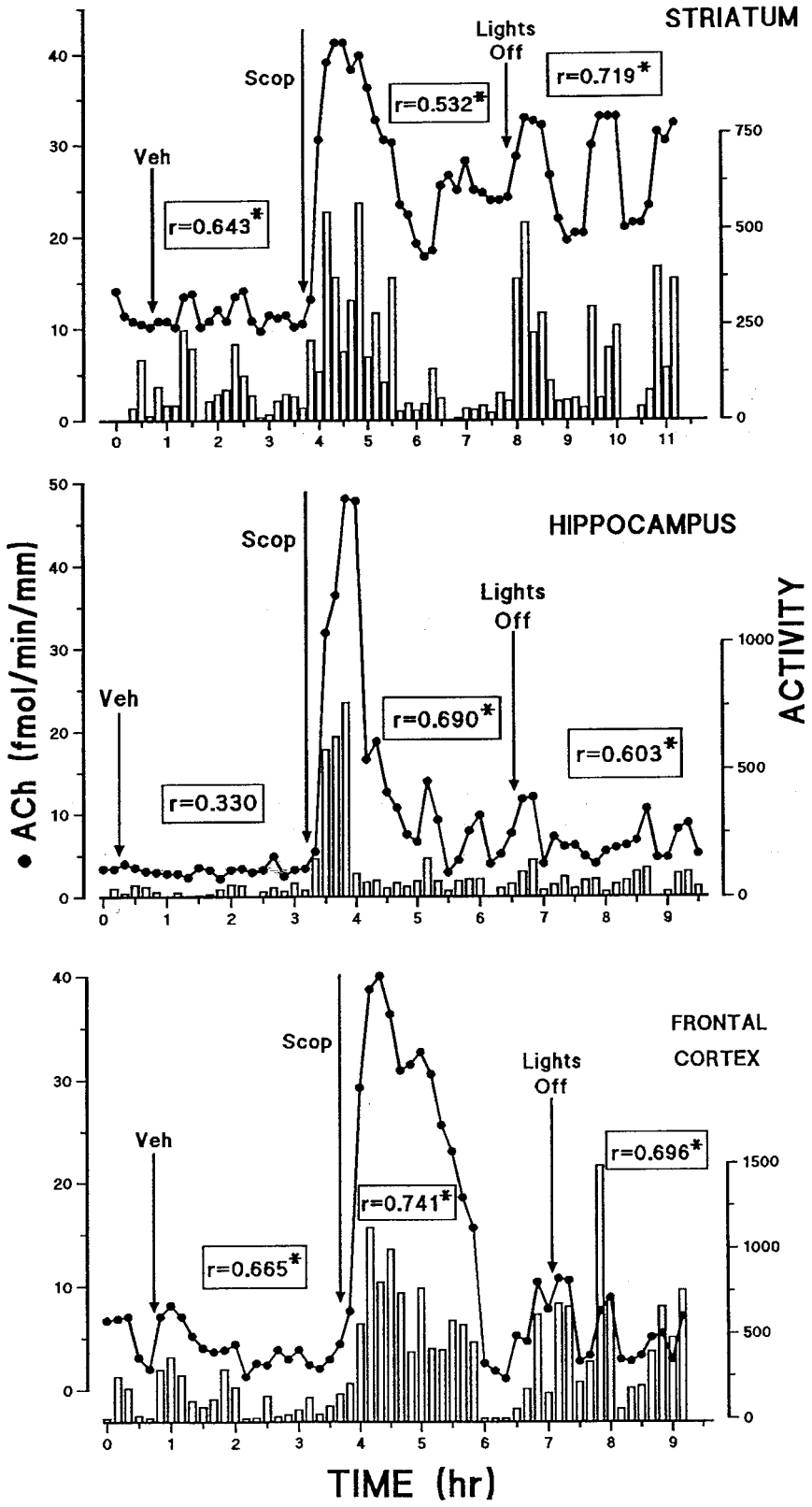


Figure 6. Dialysate ACh concentrations (closed circles) and activity counts (open bars) from three individual rats after injections of vehicle (1 ml/kg) and scopolamine (0.4 mg/kg), and exposure to dark. Insets show Pearson's correlation coefficients of the two measures for the time points within each experimental treatment. * $p<0.05$.

Table 2. Correlation of regional ACh outputs and activity counts during experimental treatments

Brain Region	Treatment		
	Vehicle	Scopolamine	Lights Off
Striatum	0.256	0.599*	0.300*
Hippocampus	0.472*	0.630*	0.595*
Frontal cortex	0.151	0.468*	0.515*

Pearson's correlation coefficients of ACh outputs (as % baseline) in the striatum, hippocampus or cortex (4-6 animals/group) and locomotor activity are shown. Analyses included the two measures for all time points (10-26) within each experimental treatment. * $p<0.05$.

correlated significantly with dialysate ACh concentrations in the striatum (4 of 5 animals, r=0.677-0.844), in the hippocampus (all 4 animals, r=0.493-0.926) and in the cortex (3 of 4 animals, r=0.696-0.899).

As evident in Table 2, when the data were grouped across animals, the correlation values became less robust. This grouping facilitates comparisons between the brain regions across the experimental conditions. The highest correlation between motor activity and ACh output occurred in the hippocampus under the scopolamine condition. The highest value for the striatum was also after scopolamine, while in the cortex ACh concentrations correlated best with motor activity during the dark phase. The lowest correlations for each group were seen after vehicle treatment.

(D) DISCUSSION

Source of microdialysate ACh and Ch

The source of ACh recovered in the microdialysates from the three studied brain regions is anatomically distinct. The striatum contains intrinsic cholinergic interneurons (Phelps *et al.*, 1985; Semba *et al.*, 1987) while the hippocampus and cortex receive cholinergic projections from the basal forebrain. Cholinergic perikarya in the basal forebrain are located among other non-cholinergic neurons in nuclei including the medial septal nucleus, the nucleus of the diagonal band of Broca, the magnocellular preoptic nucleus, substantia innominata and the globus pallidus. An alternative subdivision of these cholinergic neurons, independent of these nuclear groups and based instead on their projection patterns, has also been suggested (Mesulam *et al.*, 1983). Although theoretical subdivisions of basal forebrain cholinergic neurons are tenable, it has also been demonstrated that these neurons form an anatomical continuum (Schwaber

et al., 1987), with hippocampal and cortical cholinergic projections originating mainly in the anterior and posterior regions, respectively, of this cholinergic basal nuclear complex.

The possibility exists that extrinsic cholinergic projections from the basal forebrain are not the sole source of ACh in the rat cortex and hippocampus. Immunohistochemical studies have suggested the presence of intrinsic cholinergic neurons in these regions (see Semba and Fibiger, 1989), a finding unique to rodents. However, a sensitive *in situ* hybridization technique failed to detect mRNA for choline acetyltransferase in the rat hippocampus or cortex (Butcher *et al.*, 1992). The possibility that cholinergic interneurons are located in these regions thus remains controversial. With regard to this controversy, attempts have been made to estimate the percentage of ACh in cortical and hippocampal microdialysates derived from basal forebrain projection neurons. Mechanical deafferentations or excitotoxic lesions of cholinergic projection neurons have decreased ACh in hippocampal microdialysates by 90% (Leanza *et al.*, 1993) and in cortical microdialysates by up to 60% (Ammassari-Teule *et al.*, 1993; Herrera-Marschitz *et al.*, 1990; Rosenblad and Nilsson, 1993). Although the completeness of the lesions may be questioned, these results may also be interpreted to support the possibility that cholinergic interneurons exist in the cortex and hippocampus and contribute to microdialysate concentrations of ACh.

The source and significance of microdialysate concentrations of Ch are not well understood. Ch is both a precursor of ACh and a product of its breakdown. Further complicating any interpretation of changes in extracellular Ch is the fact that at least 80% of the Ch turnover in the brain is thought to be involved in phospholipid metabolism (Choi *et al.*, 1975). Additional investigations of the source of extracellular Ch are required before the meaning of the experimentally manipulated concentrations of dialysate Ch can be determined.

Basal concentrations of microdialysate ACh and Ch

Previous microdialysis studies comparing baseline outputs of ACh in the frontal cortex, hippocampus and striatum have reported values that are an order of magnitude higher than those reported here (Ajima and Kato, 1988; Toide and Arima, 1989; Wu *et al.*, 1988). These discrepancies are likely due to differences in probe type and brain placement, post-operative recovery times, type and concentration of the cholinesterase inhibitor, and composition and pH of the perfusate. The lower concentration of cholinesterase inhibitor used in this study, and the more physiological pH and calcium concentration of the perfusion solution are the most likely sources of the abovementioned differences in basal outputs. Indeed, the values reported here for microdialysate ACh in the individual brain regions are comparable to those reported in experiments using more similar methodologies (Damsma *et al.*, 1987b; Durkin *et al.*, 1992; Mark *et al.*, 1992).

The rank order of baseline concentrations measured in the three brain regions generally compares well with those found previously in dialysates and tissue homogenates: for ACh, striatum >> frontal cortex $\simeq$ hippocampus; for Ch, striatum > hippocampus > frontal cortex (Ajima and Kato, 1988; Jope, 1982; Toide and Arima, 1989; Wecker and Dettbarn, 1979; Wu *et al.*, 1988). In contrast, a very different rank order for basal ACh (hippocampus >> frontal cortex $\simeq$ striatum) was found in an experiment using a perfusate lacking an AChE inhibitor (Xu *et al.*, 1991). However, given that this result does not agree with earlier reports from these researchers, again using AChE inhibitor-free perfusion (Ajima and Kato, 1988), further experiments will be necessary to determine the possibly differential effects of AChE inhibition on basal dialysate concentrations of ACh in these three brain regions.

Effects of vehicle injection on ACh release

Subcutaneous injection of vehicle tended to increase locomotion and dialysate ACh concentrations in the hippocampus and frontal cortex, although the effect in the latter structure failed to reach significance due to high inter-subject variability. This finding is reliably replicated, for both cortical and hippocampal ACh release, in later chapters (Chapters III, IV, V). It has also been demonstrated that ACh release is increased in the hippocampus of rats being handled (Nilsson *et al.*, 1990). Arousing stimuli such as handling and injecting the animals may involve stress, which has also been shown to increase microdialysate concentrations of ACh in the hippocampus (Imperato *et al.*, 1992; Nilsson and Björklund, 1992). The finding that a control injection increases ACh release in the hippocampus and cortex emphasizes the necessity of including control vehicle injections when studying the effects of drugs on cholinergic activity in these regions; this finding also suggests that it may be possible to study cholinergic correlates of behaviour in one or both of these structures.

Effects of the muscarinic receptor antagonist on ACh release

It has been previously demonstrated, using *in vitro* techniques, that muscarinic receptor antagonists including scopolamine increase ACh release in the striatum (Dolezal and Wecker, 1990; Lefresne *et al.*, 1978), hippocampus (Nordstrom and Bartfai, 1980; Szerb *et al.*, 1977), and cortex (Aquilonius *et al.*, 1972; Rospars *et al.*, 1977). Such antagonists are thought to block an inhibitory feedback mechanism. This phenomenon has also been confirmed using microdialysis (de Boer *et al.*, 1990c; Marien and Richard, 1990; Toide, 1989; Toide and Arima, 1989; Watanabe and Shimizu, 1989). However, it must be noted that these dialysis experiments and those reported here have been carried out in the presence of AChE inhibition. Decreasing the degradation of ACh in the synapse with an AChE inhibitor may be expected to increase cholinergic autoreceptor occupancy and thereby alter the effects of autoreceptor ligands on ACh release. Indeed,

when an AChE inhibitor is not included in the perfusate, the muscarinic antagonist atropine does not increase microdialysate concentrations of ACh in the cortex or striatum (de Boer *et al.*, 1990b; Messamore *et al.*, 1993). In addition, the muscarinic receptor agonist oxotremorine decreases striatal ACh release in the absence, but not in the presence, of cholinesterase inhibition (de Boer *et al.*, 1990b) leading the authors of this study to conclude that under physiological conditions, muscarinic autoreceptors are not fully occupied. However, given that microdialysis depletes neurochemicals such as ACh from the sampled tissue (Benveniste, 1989), it is unclear whether perfusion without an AChE inhibitor more accurately reflects physiological conditions than does perfusion with AChE inhibitors.

The differences in magnitude of scopolamine's effect on ACh release (cortex $\simeq$ hippocampus >> striatum) may reflect regional differences in muscarinic autoreceptor location and/or function. In the cortex and hippocampus, the effect of the antagonist on ACh release is thought to be mediated by muscarinic autoreceptors located on cholinergic terminals (Marchi *et al.*, 1983; Molenaar and Polak, 1980; Sethy and Hyslop, 1990). In contrast, evoked ACh release in the striatum is apparently not controlled by muscarinic terminal autoreceptors (James and Cubeddu, 1987; Marchi *et al.*, 1983; Raiteri *et al.*, 1984). The necessary components for the effects of muscarinic antagonists on striatal ACh are known to be intrinsic to this region because intrastriatal application of antimuscarinics through the dialysis probe increases dialysate ACh concentrations (de Boer *et al.*, 1990c; Marien and Richard, 1990). The increased release of ACh measured in the striatum after scopolamine administration (this study, Toide and Arima, 1989; Watanabe and Shimizu, 1989) may therefore be mediated by multi-neuron circuits within the striatum, or possibly by dendritic or somal receptors located on the cholinergic interneurons.

Effects of the onset of dark phase on ACh release

Exposure to dark at the appropriate time in the rats' day-night cycle tended to transiently increase both locomotion and microdialysate concentrations of ACh in all three brain regions. Only the increase in the striatum was statistically significant due to the high variability of the locomotor responses and the neurochemical responses in the other two brain areas. The effect in the cortex has recently been confirmed in studies which used the onset of darkness as a stimulus to reliably increase cortical ACh release (Moore *et al.*, 1992; 1993). It has also been reported that sensory stimuli other than darkness, both tactile and non-tactile, increase hippocampal but not cortical ACh release as assessed using a superfusion technique (Dudar *et al.*, 1979). This is in contrast to the lability of cortical ACh release found here and may be due to methodological differences.

In addition to a transient increase in ACh release at the onset of the dark phase, cholinergic activity is reportedly higher on average over the course of the dark phase when compared to the light phase, as discussed below. Wholebrain ACh concentrations exhibit diurnal oscillations; the lowest concentrations of ACh in tissue extracts, thought to reflect high cholinergic activity, have been found six hours after the onset of the dark period (Hanin *et al.*, 1970). Microdialysate concentrations of ACh in the cortex (Jiménez-Capdeville and Dykes, 1993; Kametani and Kawamura, 1991) and hippocampus (Mizuno *et al.*, 1991) have also been demonstrated to be higher during the dark phase of the day/night cycle. Together, these data and the results of the experiments reported here suggest that environmental stimuli can influence cholinergic neurotransmission in the hippocampus and frontal cortex.

Correlation between ACh release and behavioural arousal after non-pharmacological stimuli

The present results indicate that changes in dialysate concentrations of ACh in the striatum, hippocampus and frontal cortex induced by the non-pharmocological stimuli correlate with locomotor activity, a measure of behavioural arousal. It should be noted that such a correlation has not been reported previously for other neurotransmitters. These findings corroborate previous suggestions of a relationship between ACh release and arousal, defined both electroencephalographically (Casamenti *et al.*, 1986; Celesia and Jasper, 1966; Kanai and Szerb, 1965; Phillis and Chong, 1965; Szerb, 1967) and behaviourally (Collier and Mitchell, 1967). ACh release has also been reported to increase in the hippocampus of swimming rats (Nilsson and Björklund, 1992) and in the hippocampus (Dudar *et al.*, 1979) and cortex (Kurosawa *et al.*, 1993) of rats walking or running on a treadmill. It should be noted, however, that it is uncertain to what degree spontaneous locomotion, which is thought to reflect an animal's level of arousal, is similar to forced locomotion, which may involve stress.

Although the accumulated evidence suggests that a correlation exists between behavioural arousal and cholinergic activity in the brain regions examined, the source of this correlation is unknown. The locomotor measure of arousal defined here can be dissociated pharmacologically from ACh release in the cortex and hippocampus: increased ACh release can occur in the absence of increased locomotion and the reverse is also true (see Chapters III, IV, V). This suggests that neither measure is absolutely necessary for the occurence of the other, although they do coincide in the non-drug situations described here. Discovering whether a cause-effect relationship is responsible for the observed correlation between behavioural arousal and ACh release, or on what common element both measures are dependent, is a challenge for future research. To accomplish this goal, more detailed behavioural descriptions, coupled with an *in vivo* ACh measurement with improved temporal resolution will be required.

Correlation between ACh release and behavioural arousal after scopolamine administration

The positive correlations between locomotor activity counts and ACh release in the three brain regions after scopolamine administration extend previous findings that muscarinic receptor antagonist-induced increases in motor activity are associated with increases in frontal and hippocampal dialysate ACh (Toide, 1989), and with decreases in wholebrain tissue levels of ACh (Frances and Jacob, 1971).

The correlation evident after application of scopolamine may be qualitatively different than that discussed above, for the non-pharmacological stimuli. In drug-free conditions, increased ACh release is associated with increased cholinergic neurotransmission; the correlation between ACh release and behavioural arousal would therefore suggest a correlation between cholinergic transmission and behavioural arousal. After injection of scopolamine, however, the increased ACh release occurs as a result of, and during, muscarinic receptor blockade. The behavioural hyperactivity caused by this anticholinergic agent thus occurs in the presence of decreased muscarinic cholinergic transmission. Therefore, while behavioural arousal correlates with cholinergic transmission after the non-pharmacological stimuli, it does not appear to be associated with muscarinic cholinergic transmission in scopolamine-treated rats. This apparent inconsistency may be accounted for in several ways: 1) Pre- and postsynaptic muscarinic receptors may differ such that the dose of scopolamine used here might preferentially bind the presynaptic autoreceptors, resulting in increased ACh release, while the postsynaptic receptors remain unblocked. This would serve to increase cholinergic transmission. This possibility can be rejected, however, as Szerb *et al.* (1977) have reported that scopolamine has a ten fold lower efficacy at presynaptic than at postsynaptic muscarinic receptors. 2) While scopolamine blocks muscarinic transmission, it would at the same time increase nicotinic cholinergic transmission by increasing ACh release. The results obtained here could be explained if the action of ACh on nicotinic receptors was responsible for the correlation between ACh release and behavioural

arousal. 3) As discussed above, the source of the correlation after the non-pharmacological stimuli is not known, and it is possible to pharmacologically dissociate this correlation. This additional example of a pharmacological dissociation suggests that the correlation does not involve a direct cause-effect relationship between ACh release and behavioural arousal. Thus, pharmacological agents such as scopolamine, which increase locomotion while decreasing muscarinic tone, may affect these two measures independently, perhaps by acting at different loci within the central and peripheral nervous systems.

Significance of the correlation between behavioural arousal and ACh release in the cortex, hippocampus and striatum

Given the previously suggested involvement of basal forebrain cholinergic neurons in arousal (see Semba, 1991), the responsiveness of ACh release in the frontal cortex and hippocampus to arousing stimuli and the correlation of this release with a behavioural measure of arousal is not surprising. The correlation of ACh release in the striatum with behavioural arousal is perhaps more unexpected. In addition to the anatomical differences between cholinergic interneurons and basal forebrain cholinergic neurons, the functions usually ascribed to the striatum are quite different from those of the hippocampus and cortex. The striatum is part of the basal ganglia, a group of nuclei traditionally thought to be involved in initiating and modulating motor output. However, the striatum is considered the most cognitive of the basal ganglia nuclei, being closely connected with the cortex. Indeed, mnemonic functions of the striatum have been suggested (Divac, 1972; Mishkin and Petri, 1984; Packard and McGaugh, 1992).

In summary, these experiments illustrate the feasibility of carrying out ACh microdialysis experiments of long duration, coupled to behavioural monitoring, in different brain regions. The data reported here indicate that arousing stimuli can increase cholinergic transmission in a regionally selective manner.

III. DOPAMINERGIC REGULATION OF CORTICAL ACETYLCHOLINE RELEASE

(A) INTRODUCTION

The extrinsic cholinergic innervation of the cortex and hippocampus emanates from posterior and anterior portions, respectively, of the cholinergic basal nuclear complex (CBC). The CBC is an anatomical continuum of cholinergic neurons which are interspersed among non-cholinergic neurons within several classically defined nuclei including the medial septal nucleus, the nucleus of the diagonal band of Broca, the magnocellular preoptic area, the substantia innominata and the globus pallidus (Schwaber *et al.*, 1987; Semba and Fibiger, 1989). Direct synaptic contacts made with these ChAT-containing neurons are relatively sparse and generally localized to distal dendrites (Ingham *et al.*, 1985). However, afferents to the CBC arise from many brain regions, as outlined below, suggesting that hippocampally- and cortically-projecting cholinergic neurons may be weakly innervated by multiple sources.

The CBC receives many inputs among which are reciprocal projections from the cortex and hippocampus, as well as ascending projections from the hypothalamus and brainstem nuclei including the ventral tegmental area, substantia nigra, dorsal and median raphe, locus coeruleus, pedunculopontine and laterdorsal tegmental nuclei (Semba and Fibiger, 1989; Woolf, 1991). The neurotransmitter contents of some of these afferents have been identified immunohistochemically. Catecholaminergic neurons defined by the presence of tyrosine hydroxylase (TH) have been retrogradely labelled, from the CBC, in regions known to contain dopaminergic and noradrenergic perikarya including the ventral tegmental area, substantia nigra and locus coeruleus (Jones and Cuello, 1989; Semba *et al.*, 1988). Serotonin-containing neurons localized in several nuclei including the dorsal raphe, and ChAT-positive neurons in the mesopontine tegmentum were also retrogradely labelled in these experiments.

Ultrastructural characterizations using electron microscopy have provided evidence of direct synaptic contacts with basal forebrain neurons. Terminals immunoreactive for TH and ChAT synapse on ChAT-labeled neurons in the CBC (Milner, 1991). Serotonin-containing terminals synapse on hippocampally-projecting basal forebrain neurons, some of which may be cholinergic (Milner and Veznedaroglu, 1993). Synaptic contacts have also been demonstrated between GABAergic terminals and ChAT containing neurons (Tóth *et al.*, 1993; Záborszky *et al.*, 1986b), or presumably cholinergic cortically-projecting neurons (Ingham *et al.*, 1988) in the basal forebrain. GABAergic synapses may arise from projections of the nucleus accumbens (Walaas and Fonnum, 1979) or hippocampus (Tóth *et al.*, 1993) or from intrinsic GABAergic neurons (Köhler and Chan-Palay, 1983; Záborszky *et al.*, 1986a). Recent electrophysiological studies of identified cholinergic neurons in the basal forebrain of guinea pig brain slices also provide evidence of GABAergic, noradrenergic, as well as histaminergic effects on the firing rates of CBC neurons (Fort *et al.*, 1993; Khateb *et al.*, 1993; Pegna *et al.*, 1993).

Presumptive glutamate/aspartate projections to the basal forebrain from many brain regions including the amygdala, thalamus and cortex have also been described (Carnes *et al.*, 1990), although neurons using excitatory amino acid transmitters are difficult to characterize anatomically. Evidence supporting a glutamatergic innervation of the CBC includes the finding that *l*-glutamate binding sites represent a large portion of the total binding sites autoradiographically identified in the basal forebrain of the rat (Zilles *et al.*, 1991) and that the AMPA subtype of glutamate receptor has been immunocytochemically localized to cholinergic basal forebrain neurons in the monkey (Martin *et al.*, 1993). In addition, Rasmusson *et al.* (1994) have recently reported that increases in cortical ACh release caused by brainstem stimulation in anaesthetized rats can be blocked by application of a glutamate antagonist into the basal forebrain. With regard to the abovementioned proposition that the presence of appropriate receptors further supports the regulatory role of a transmitter within a certain brain region, it

should be noted that receptors for ACh, serotonin, GABA, dopamine (DA) and noradrenaline have also been autoradiographically identified in the basal forebrain (Zilles *et al.*, 1991). Evidence of whether some of these receptors may be situated on cholinergic neurons awaits further ultrastructural studies.

In addition to the classical neurotransmitters, a variety of neuropeptide transmitters and growth factors may regulate CBC neurons. There is evidence for interactions between basal forebrain neurons, in some cases identified as cholinergic, and terminals containing substance P, enkephalin, somatostatin, neuropepetide Y, neurotensin or vasopressin (reviewed by Záborszky *et al.*, 1991). In addition, receptors for neurotrophins and estrogen have been located in CBC neurons (Toran-Allerand *et al.*, 1992).

Considered together, the above anatomical data suggest that the activity of CBC neurons may be regulated by a great variety of neurotransmitters. The regulation of these cholinergic neurons, specifically those projecting to the cortex, by the neurotransmitter DA has been investigated in the experiments discussed in this chapter. To this end, *in vivo* dialysate concentrations of ACh from the frontal cortex of conscious, freely moving rats were measured after systemic administration of the psychomotor stimulant *d*-amphetamine (AMPH) or the DA receptor agonist apomorphine, and after local application of AMPH through the dialysis probe. To determine which DA receptor subtype(s) may mediate the dopaminergic regulation of cortically-projecting cholinergic neurons, the effects of selective D_1-like and D_2-like receptor agonists on cortical ACh release were examined. In addition, the extent to which selective D_1-like and D_2-like receptor antagonists attenuate AMPH- and apomorphine-induced effects on cortical ACh release was determined. Further references to "D_1" and "D_2" should be interpreted as "D_1-like" and "D_2-like" respectively (see Discussion).

(B) MATERIALS AND METHODS

Experimental protocol and drugs

The size and strain of subject rats were as in Chapter II. Dialysis experiments were conducted as described in Chapter II and involved the subcutaneous (s.c.) injection of the following drugs, alone or in combination: *d*-amphetamine sulfate (AMPH, 2.0 mg/kg; BDH), apomorphine hydrochloride (1.0 mg/kg; Sigma), CY 208-243 (1.0 mg/kg containing 0.4% acetic acid; Sandoz), quinpirole hydrochloride (0.2 or 0.5 mg/kg; RBI), (+)-4-propyl-9-hydroxynaphthoxazine (PHNO, 0.05 mg/kg; Merck, Sharp and Dohme), haloperidol base (0.15 mg/kg; McNeil Pharmaceutical), raclopride (1.0 mg/kg; Astra), and SCH 23390 (0.3 mg/kg; Schering Plough). The drugs were dissolved in distilled water, and injected in a volume of 1 ml/kg. AMPH (10 *u*M) was also administered directly through the dialysis probe by its addition to the perfusion solution. The doses used in this study have significant effects on behaviour (Christensen *et al.*, 1984; Markstein *et al.*, 1988; Martin *et al.*, 1984; Ogren *et al.*, 1986) and/or have reliable neurochemical effects on dopaminergic systems (Imperato and DiChiara, 1985; Kuczenski and Segal, 1989; Sharp *et al.*, 1987) or on striatal cholinergic neurons (Bertorelli and Consolo, 1990; Damsma *et al.*, 1990; 1991; Robertson *et al.*, 1993; Westerink *et al.*, 1990).

Surgery and microdialysis

Transverse dialysis probes were stereotaxically implanted into the frontal cortex of rats as described in Chapter II. Microdialysis was carried out as previously described with the following exception: the probe outlet was connected to the sample valve by a length of polyethylene tubing, rather than fused silica tubing, containing 50 *u*l.

Assay of ACh

ACh was assayed by HPLC-ECD as described in Chapter II.

Statistical analyses

Biochemical data were calculated as a percent of baseline concentrations, 100% baseline being defined as the average of the last three pre-drug values. Univariate ANOVAs with repeated measures were used to compare the effects of the different drugs on dialysate ACh concentrations. Reported values refer to the main group effect of the experimental treatment, unless otherwise noted as referring to the interaction effect of time with experimental treatment. In the latter case, Huynh-Feldt adjustments of degrees of freedom were made to account for the use of time as a repeated measure. For the local application of AMPH, the last baseline sample and the six samples during AMPH perfusion were used to test for an effect of the drug over time.

(C) RESULTS

The average baseline output of ACh (± S.E.M.) from the frontal cortex in all of the animals was 46.66 ± 6.69 fmol/min (n=88). Systemically administered AMPH (2.0 mg/kg) increased ACh output to 280% of baseline values within 1 h and the increase lasted for approximately 3 h. This was statistically significant [$F(1,10)=46.98$, $p<0.001$] compared to the transient increase after injection of vehicle (149%, Fig. 7A). The DA receptor agonist apomorphine (1.0 mg/kg) also significantly increased ACh for 1 h to a maximum of 220% baseline [$F(1,9)=5.73$, $p=0.04$; Fig. 7A]. Both AMPH and apomorphine caused behavioural hyperactivity (data not shown). Local application of AMPH (10 *u*M) through the dialysis probe did not influence behavioural activity and did not significantly affect interstitial concentrations of ACh in the frontal cortex [$F(3.59,10.76)=0.54$; Fig. 7B].

The effects of selective DA receptor agonists on cortical dialysate output of ACh are shown in Figs. 8 and 9. The effect of the specific D_2 agonist quinpirole, at 0.2 or

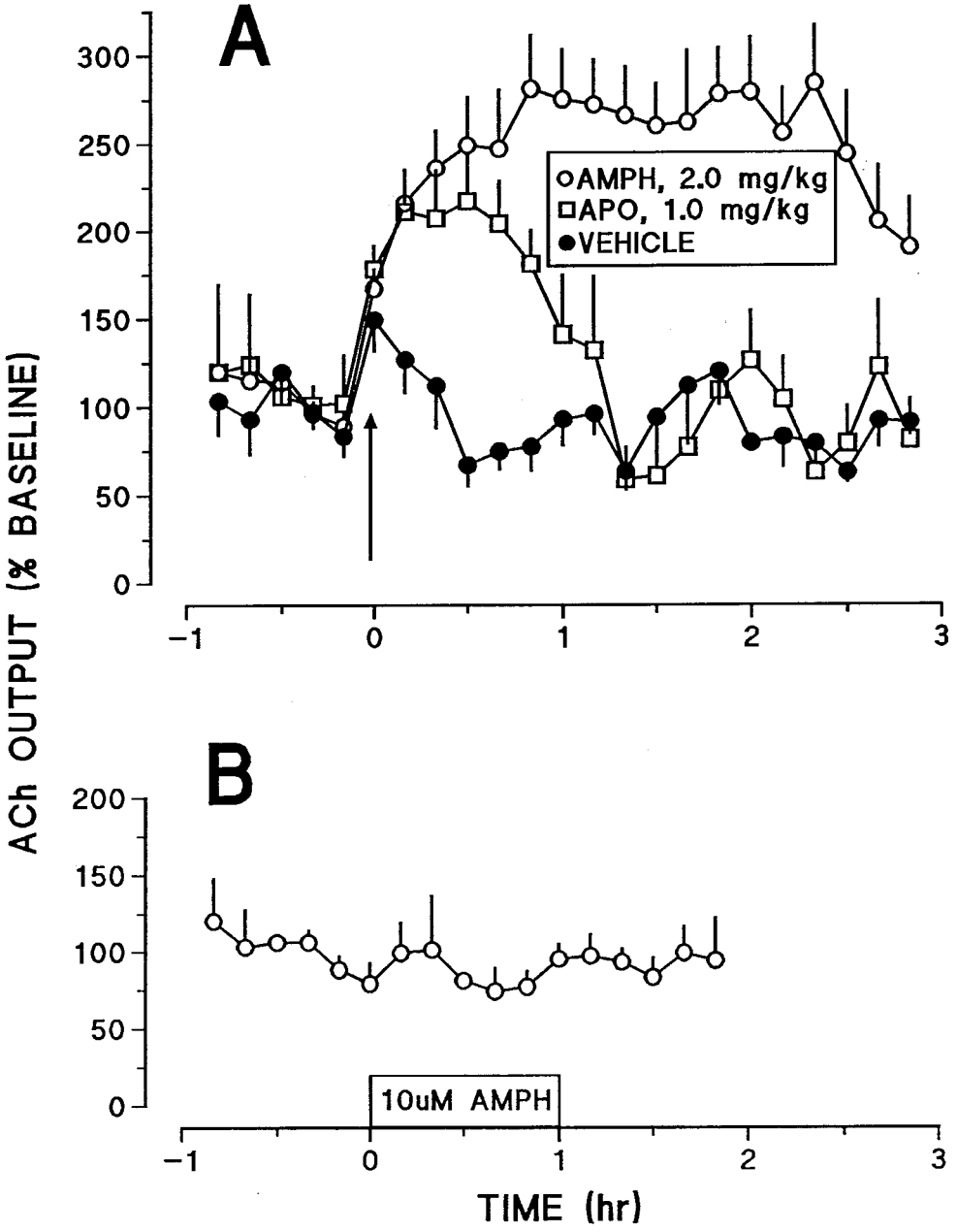


Figure 7. Frontal cortex dialysate values of ACh after A: injections of *d*-amphetamine (AMPH; 2.0 mg/kg; open circles), apomorphine (APO; 1.0 mg/kg; open squares), or vehicle (1.0 ml/kg; closed circles), and B: local administration of AMPH (10 μM) through the dialysis probe. Data points represent group means (n=4-6) ± S.E.M.

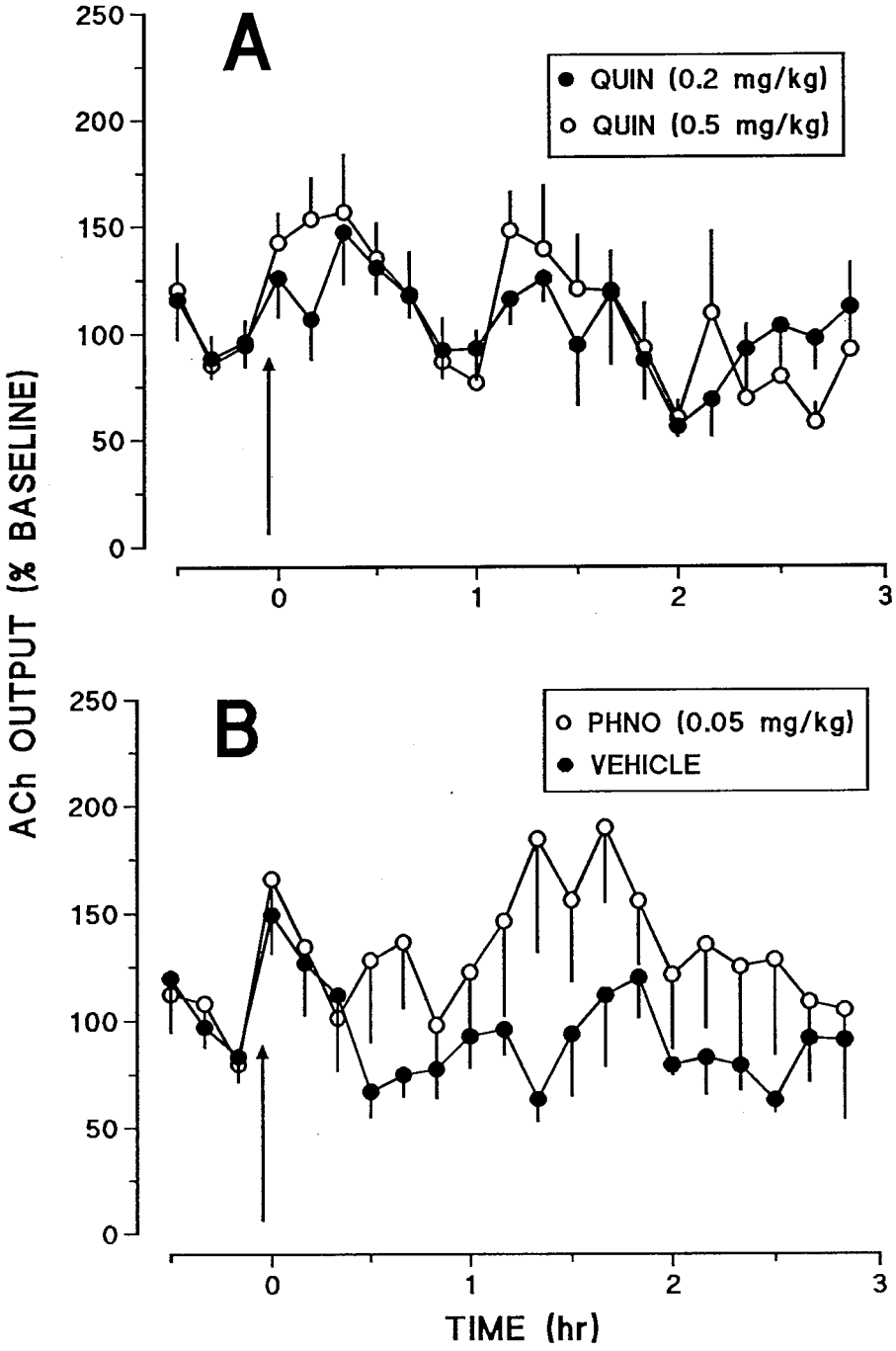


Figure 8. Frontal cortex dialysate values of ACh after injections of A: quinpirole (QUIN) 0.2 mg/kg (closed circles) or 0.5 mg/kg (open circles), and B: PHNO (0.05 mg/kg; open circles) or vehicle (1.0 ml/kg; closed circles). Data points represent group means (n=5-7) ± S.E.M.

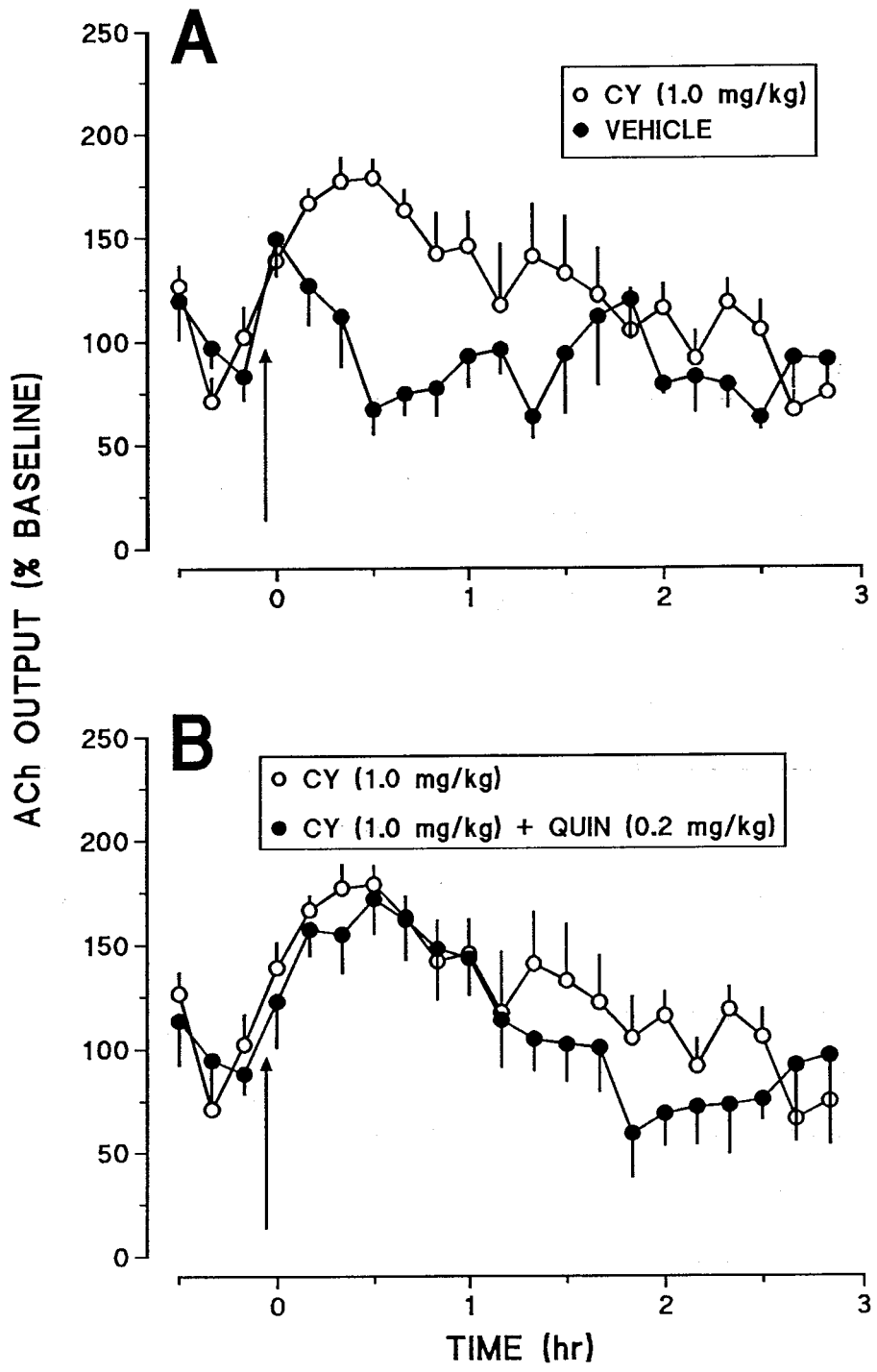


Figure 9. Frontal cortex dialysate values of ACh after injections of CY 208-243 (CY; 1.0 mg/kg; open circles in A and B) or a combination of quinpirole (QUIN; 0.2 mg/kg) and CY (1.0 mg/kg; closed circles in B). The effect of vehicle injection from Fig. 7A is included in A (closed circles) for comparison. Data points represent group means (n=5-6) ± S.E.M.

0.5 mg/kg, was not significantly different from that of vehicle injections [$F(1,10)=0.97$, and $F(1,9)=1.03$, respectively; Fig. 8A]. Similarly, the D_2 agonist PHNO (0.05 mg/kg) did not have a significant effect on ACh output compared to vehicle-treated animals [$F(1,11)=2.13$; Fig. 8B]. Although a tendency for increasing ACh values was evident 1 hour after administration of PHNO, the effect failed to reach significance due to high variability in these drug-treated animals. Although the D_2 agonists did not affect cortical ACh release, these drugs produced behavioural stereotypies that lasted for approximately 1.5 h.

The D_1 receptor agonist CY 208-243 (1.0 mg/kg) significantly increased ACh concentrations to 180% of baseline within 30 min, and this lasted approximately 1 h compared to the transient increase observed after vehicle injections [$F(1,9)=8.39$, $p=0.018$; Fig. 9A]. This drug treatment did not appreciably increase the rats' locomotion. The D_1 agonist-induced increase in cortical ACh release was not altered by simultaneous administration of the D_2 agonist quinpirole [0.2 mg/kg; $F(1,8)=1.00$; Fig. 9B]. The behavioural effects of the co-administered D_1 and D_2 receptor agonists were similar to that of the D_2 agonist alone.

Figs. 10 and 11 show the effects of the DA receptor antagonists on ACh efflux and on the ACh response to the subsequently administered AMPH. (The AMPH response from Fig. 7A is superimposed for comparison). Compared to the effect of the vehicle injection which is depicted in Fig. 7A, the selective D_1 antagonist SCH 23390 (0.3 mg/kg) maximally decreased ACh to 30% of baseline values [$F(1,9)=22.27$, $p=0.001$; Fig. 10]. When animals received AMPH 30 min after SCH 23390, the ACh response was significantly lower than that after AMPH alone [130% vs 280% maximal values; $F=(1,9)=42.82$, $p<0.001$], significantly higher than that after SCH 23390 alone [$F(1,8)=16.63$, $p=0.004$; Fig. 10], and not significantly different from that after the vehicle injection [$F(1,9)=0.13$].

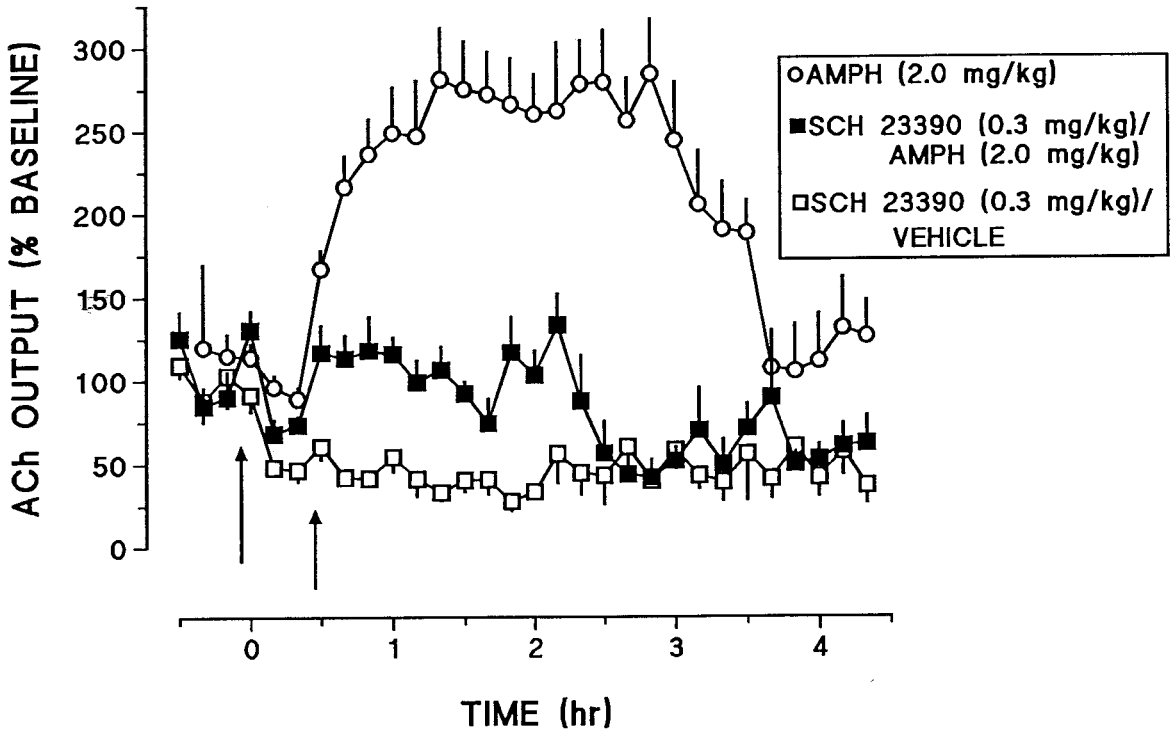


Figure 10. Frontal cortex dialysate values of ACh. SCH 23390 (0.3 mg/kg) was injected 30 min prior to peripheral administration of *d*-amphetamine (AMPH; 2.0 mg/kg; closed squares) or vehicle (1.0 ml/kg; open squares). The effect of AMPH alone (open circles) from Fig. 7A is included for comparison. Data points represent group means (n=5-6) ± S.E.M.

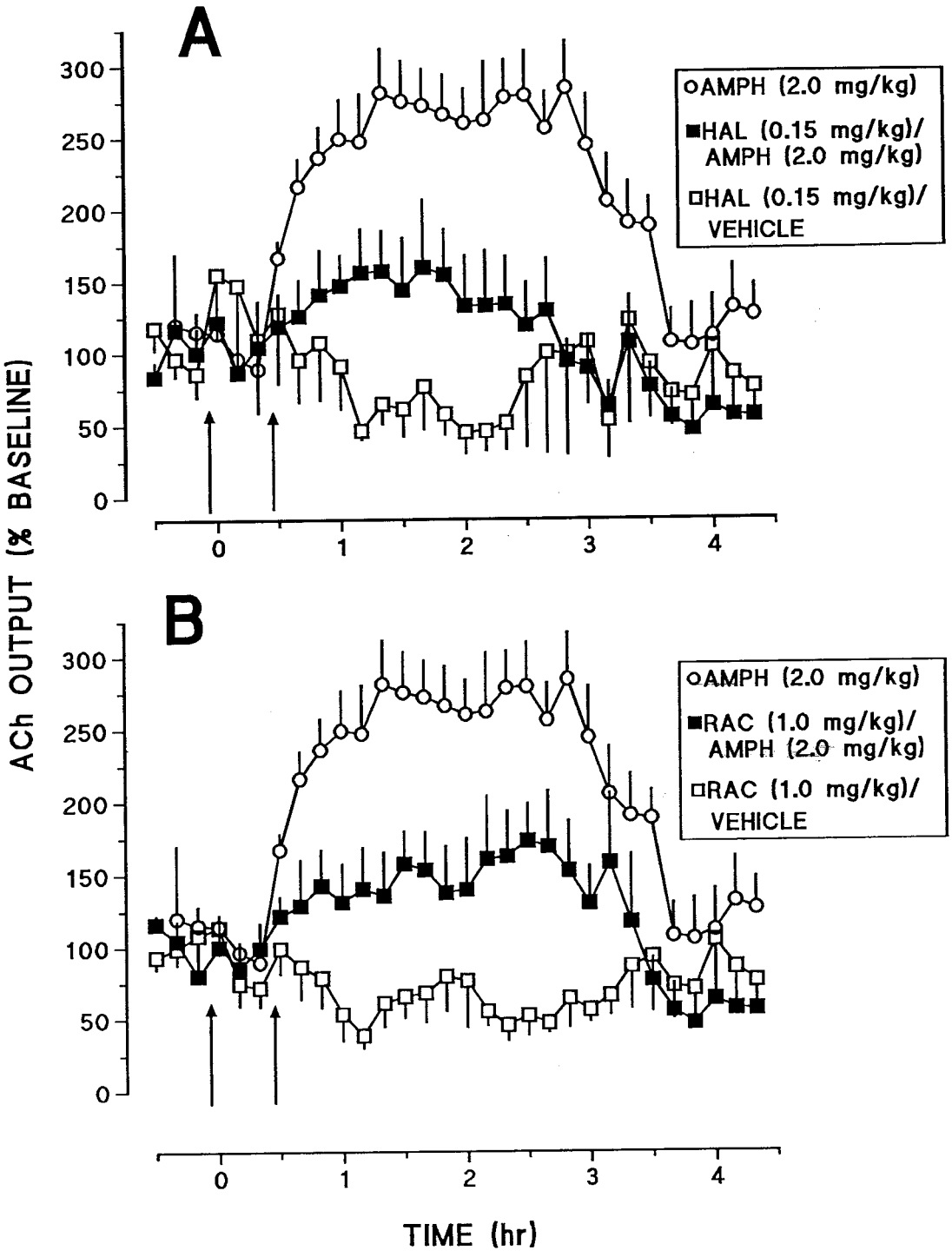


Figure 11. Frontal cortex dialysate values of ACh. A: Haloperidol (HAL; 0.15 mg/kg) and B: raclopride (RAC; 1.0 mg/kg) were injected 30 min prior to peripheral adminstration of *d*-amphetamine (AMPH; 2.0 mg/kg; closed squares) or vehicle (1.0 ml/kg; open squares). The effect of AMPH alone (open circles) from Fig. 7A is included for comparison. Data points represent group means (n=5-6) ± S.E.M.

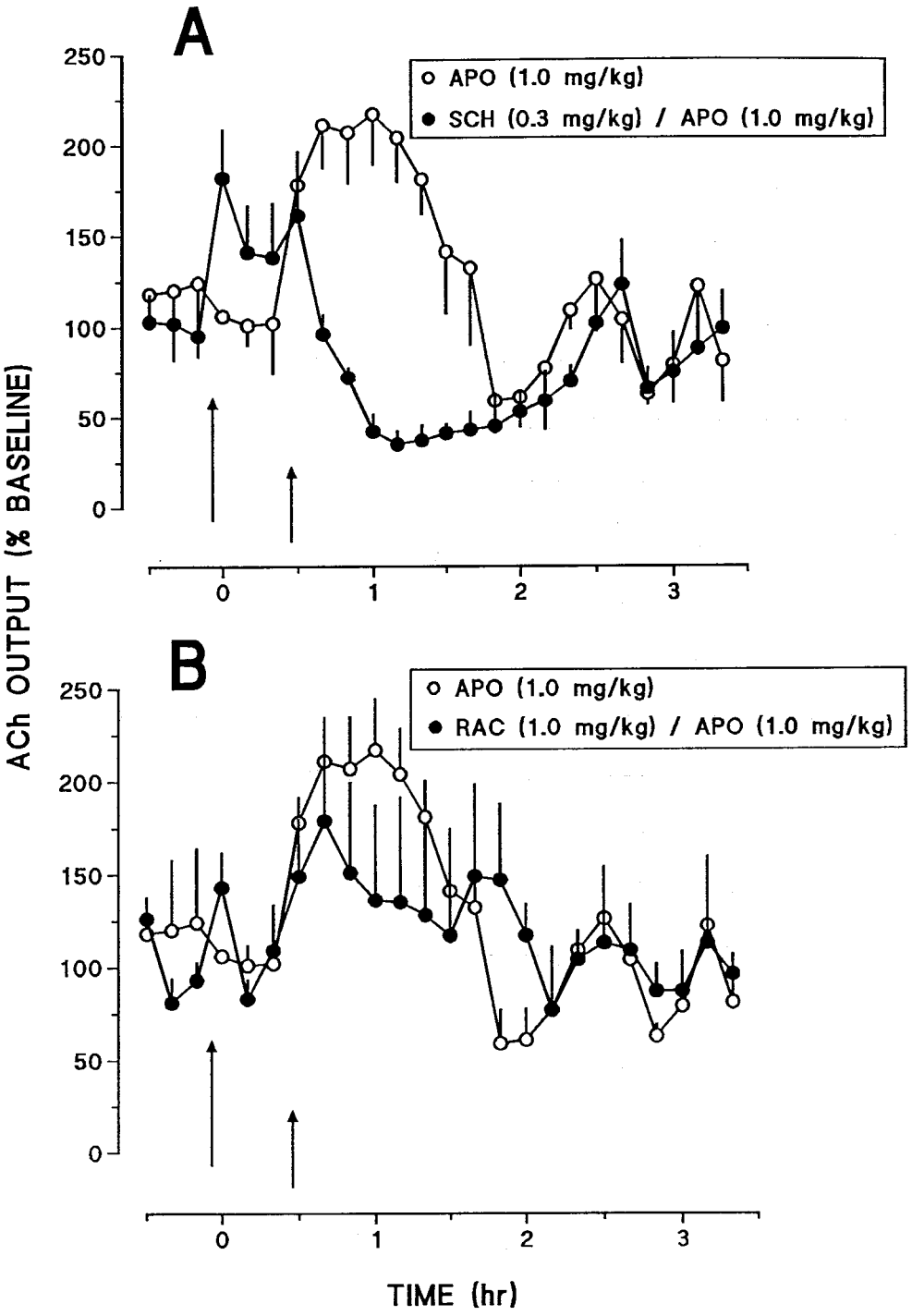


Figure 12. Frontal cortex dialysate values of ACh. A: SCH 23390 (SCH; 0.3 mg/kg) and B: raclopride (RAC; 1.0 mg/kg) were injected 30 min prior to peripheral administration of apomorphine (APO; 1.0 mg/kg; closed circles). The effect of APO alone (open circles) is included for comparison. Data points represent group means (n=5-6) ± S.E.M.

The somewhat selective D_2 receptor antagonist haloperidol (0.15 mg/kg) and the selective antagonist raclopride (1.0 mg/kg) did not significantly affect dialysate concentrations of ACh [$F(1,9)=0.04$, $F(1,9)=3.42$, respectively; Fig. 11], although a tendency for both to result in decreasing concentrations was apparent. When AMPH was administered 30 min after the neuroleptics, the ACh response was less than that of AMPH alone and greater than the neuroleptic alone. Haloperidol did not completely block the effect of AMPH, because AMPH significantly increased ACh output after haloperidol, compared to haloperidol alone [drug x time interaction: $F(5.48,43.81)=2.54$, $p=0.038$; the overlapping curve shapes yielded a nonsignificant main effect: $F(1,8)=0.42$; Fig. 11A]. Raclopride also did not completely block the effect of AMPH, because AMPH administration after raclopride caused a significant increase over raclopride alone [$F(1,8)=6.01$, $p=0.04$; Fig. 11B]. However, both D_2 antagonists did significantly attenuate the ACh response to AMPH, compared to the response of AMPH alone [$F(1,9)=16.53$, $p=0.003$ for haloperidol and $F(1,9)=8.64$, $p=0.017$ for raclopride]. The maximum increase after AMPH (280%) was decreased to 160% by haloperidol and 170% by raclopride. The attenuated responses of ACh to AMPH injections after the neuroleptics did not differ significantly from the vehicle condition [$F(1,9)=2.16$ for haloperidol and $F(1,9)=3.50$ for raclopride]. All three receptor antagonists completely blocked the AMPH-induced behavioural hyperactivity (data not shown).

Fig. 12 shows the effects of selective DA receptor antagonists on the ability of apomorphine, the non-selective DA receptor agonist, to increase ACh release. (The apomorphine response from Fig. 7A is superimposed for comparison). The apomorphine-induced increase in ACh output was completely blocked by the D_1 receptor antagonist SCH 23390 [0.3 mg/kg; $F(1,8)=10.22$, $p=0.013$; Fig. 12A]. Although the D_2 receptor antagonist raclopride (1.0 mg/kg) appeared to partially block the apomorphine-induced increase in cortical ACh release, this effect was not statistically

significant [F(1,9)=0.02; Fig. 12B]. Both the D_1 and D_2 receptor antagonists blocked apomorphine-induced behavioural activation.

(D) DISCUSSION

The results presented here, that an indirect DA agonist (AMPH) and a DA receptor agonist (apomorphine) increase cortical ACh release, suggest that DA regulates the activity of cortically-projecting cholinergic neurons. This effect is probably mediated outside of the cortex given that cortically applied AMPH did not increase ACh release. Because D_1 selective agonists and antagonists more robustly affected basal and drug-stimulated release of ACh than did D_2 selective drugs, dopaminergic regulation of cortical ACh release is apparently mediated primarily via D_1 receptors, although a contribution of D_2 receptors cannot be excluded.

Regulation of cortically-projecting cholinergic neurons by DA

The results summarized above confirm previous evidence that apomorphine and AMPH increase cortical ACh release as assessed using the cortical cup technique (Beani and Bianchi, 1973; Casamenti *et al.*, 1986; 1987; Hemsworth and Neal, 1968; Mantovani *et al.*, 1977; Pepeu and Bartolini, 1968). A microdialysis study has also confirmed this effect of AMPH on cortical ACh release (Okada, 1991). In addition, AMPH has been reported to increase the turnover rate of cortical ACh although apomorphine does not (Robinson *et al.*, 1978) suggesting either that the latter measure is not as sensitive as microdialysis or that the turnover of ACh is not always coupled to ACh release.

AMPH is a psychomotor stimulant which induces behavioural hyperactivity and EEG desynchronization (Fairchild *et al.*, 1967). The behavioural effects of this drug are usually ascribed to its ability to elevate synaptic concentrations of DA, noradrenaline

(NA) and, to a lesser extent, serotonin (5-HT) (Azzaro and Rutledge, 1973; Kuczenski, 1983). The data presented here demonstrate that the AMPH-induced increases in monoaminergic transmission have robust effects on cortical ACh release. It is noteworthy that AMPH's ability to desynchronize cortical EEG activity is, at least in part, cholinergically mediated (Dren and Domino, 1968; White and Daigneault, 1959). DA could be responsible for AMPH's stimulation of cortical ACh release given that the DA receptor agonist apomorphine, which increases the firing rate of unidentified neurons in the basal forebrain (Napier *et al.*, 1991), increases cortical ACh release (present results; Mantovani *et al.*, 1977).

The other monoamines that are affected by AMPH, NA and 5-HT, have not been examined in these experiments, but existing evidence suggests that these neurotransmitters are less likely candidates for mediating the AMPH-induced increases in cortical ACh release. For example, while AMPH increases synaptic concentrations of NE, this transmitter appears to inhibit cortical ACh release. Application of NA or stimulation of noradrenergic cell bodies in the locus coeruleus decreases cortical release of ACh, apparently *via* actions on alpha receptors (Beani *et al.*, 1978; Bianchi *et al.*, 1979; Vizi, 1980). Conversely, while alpha antagonists have been reported to block AMPH-induced increases in cortical ACh turnover (Robinson *et al.*, 1978), Bartolini and Pepeu (1970) found that beta, but not alpha, blockers attenuated the effect of AMPH on cortical ACh release. An understanding of these apparently contradictory results awaits further study using the more selective drugs and the more sensitive procedures that are now available.

AMPH also increases synaptic concentrations of 5-HT (Kuczenski and Segal, 1989) but like NE, 5-HT has previously been reported to have an inhibitory influence on cortical ACh release as determined in slice, synaptosome or cortical cup preparations (Barnes *et al.*, 1989; Bianchi *et al.*, 1990; Muramatsu *et al.*, 1990; Siniscalchi *et al.*, 1990; 1991). Recent microdialysis results have revealed, however, that increasing serotonergic

tone *via* administration of the 5-HT releasing agent fenfluramine increases cortical ACh release (Hirano, Day and Fibiger, submitted). Regardless of whether 5-HT may also regulate CBC neurons in an excitatory manner, the near complete blockade of the AMPH-induced increases in cortical ACh release by the D_1 antagonist suggests that 5-HT does not mediate AMPH's effect on CBC neurons. In support of the conclusion that DA is the primary transmitter through which AMPH increases cortical ACh release, Casamenti *et al.* (1987) have reported that AMPH-induced increases in cortical ACh release are not reduced by the 5-HT synthesis inhibitor, para-chlorophenylalanine, or the NA depletor, N-(2-chloroethyl)-N-ethyl-bromobenzylamine (DSP4).

Neuroanatomy of dopaminergic regulation of cortically-projecting cholinergic neurons

The site of interaction between dopaminergic drugs and cortically-projecting cholinergic neurons has not been determined by these experiments. As outlined in the Introduction, direct synaptic contact between catecholaminergic terminals and cholinergic perikarya in the CBC has been reported (Milner, 1991). Given that catecholaminergic innervation of the basal forebrain arises in part from nuclei containing dopaminergic perikarya (Jones and Cuello, 1989; Semba *et al.*, 1988), it is possible that at least a portion of this innervation is dopaminergic. Indirect support of a dopaminergic interaction with cholinergic perikarya includes the findings that unidentified neurons in the basal forebrain exhibit increased firing rates in response to microiontophoretic application of DA (Napier *et al.*, 1991) and receptors for DA are present in the basal forebrain (Zilles *et al.*, 1991). Although forebrain cholinergic neurons apparently do not express D_2 receptor mRNA (Le Moine *et al.*, 1990), parallel evidence for other DA receptor subtypes is unavailable.

The possibility also exists that dopaminergic regulation of cortical ACh release is mediated by presynaptic control of cholinergic terminals in the cortex. Neuroanatomical studies have demonstrated a dopaminergic innervation of, and DA receptors in, the

cortex (Lindvall *et al.*, 1974; Richfield *et al.*, 1989). However, the data presented here indicate that AMPH does not act locally in the frontal cortex to increase ACh release and this is supported by previous findings (Beani and Bianchi, 1973; Pepeu and Bartolini, 1968). It is unlikely that the lack of effect of locally applied AMPH is due to insufficient dosage: AMPH is known to be permeable through the dialysis membrane used here (Nomikos *et al.*, 1990), and the concentration of AMPH chosen for this experiment has potent effects on dialysate concentrations of both DA and ACh in the striatum, as shown previously using identical methodology (Westerink *et al.*, 1990). Future experiments using more selective dopaminergic drugs may help determine if cortical DA can presynaptically regulate cortical ACh release.

It is also possible that multisynaptic mechanisms mediate DA's effects on cortically-projecting cholinergic neurons. For example, DA in the nucleus accumbens may serve to disinhibit CBC neurons by inhibiting the activity of a GABA-containing projection from the nucleus accumbens to the ventral pallidum (Casamenti *et al.*, 1986). Previous evidence supporting this possibility includes: 1) a projection from the nucleus accumbens terminates on CBC neurons (Záborszky and Cullinan, 1992); 2) this projection may use GABA as a transmitter (Walaas and Fonnum, 1979); 3) peripheral administration of a dopaminergic agonist decreases extracellular concentrations of GABA in the basal forebrain (Bourdelais and Kalivas, 1992); 4) microinjections of DA into the nucleus accumbens increases the firing rate of the same unidentified basal forebrain neurons that are stimulated by a GABA antagonist (Yang and Mogenson, 1989); and 5) GABA regulates cortically-projecting cholinergic neurons (Sarter *et al.*, 1990).

Previously, studies of interactions between DA and ACh have focussed mainly on the striatum (Lehmann and Langer, 1983; Stoof *et al.*, 1992) largely due to the putative clinical relevance of such interactions to Parkinson's disease and neuroleptic-induced extrapyramidal side effects (Barbeau, 1962; Borison and Diamond, 1987; McGeer *et al.*, 1961). Abundant evidence from striatal slice and homogenate studies has shown that

local DA receptors of the D_2 subtype have inhibitory effects on striatal ACh release (Scatton, 1982; Stoof and Kebabian, 1982). Thus, at a superficial level, an excitatory dopaminergic regulation of cortical ACh release might be unexpected based on the previously reported inhibitory regulation of striatal ACh by DA. However, when examined in intact preparations using *in vivo* microdialysis, the effect of DA on striatal ACh release has recently been shown to be more complex than earlier reports had suggested: excitatory actions at DA receptors of the D_1 subtype appear to override local D_2 mediated inhibitory actions of DA. There remains a controversy as to whether the D_1 receptors mediating this effect on striatal ACh release are located in the striatum (Consolo et al., 1992; Zocchi and Pert, 1993) or extrastriatally (Damsma *et al.*, 1991). The results presented here suggesting that extracortical dopamine receptors stimulate cortical ACh release are not dissimilar to the suggestion that extrastriatal D_1 receptors stimulate striatal ACh release.

DA receptor subtype(s) mediating dopaminergic regulation of cortically-projecting cholinergic neurons

Receptors for DA were subdivided in the early 1980's into two classes based on their pharmacological, biochemical and physiological properties (Kebabian and Calne, 1979). More recently, gene cloning techniques have led to the subdivision of DA receptors into five genetically-defined subtypes. The D_5 receptor is similar to the D_1 receptor, while the D_3 and D_4 receptors are more similar to the previously defined D_2 receptor. For example, quinpirole was previously described as a D_2 agonist, but has been shown to have high affinity at the D_3 and D_4 sites (Sokoloff *et al.*, 1990; Tang *et al.*, 1994). PHNO has been reported to bind D_2 rather than D_3 or D_4 receptors (Seeman *et al.*, 1993). Studies of the D_3, D_4 and D_5 receptors are in the early stages, and the experiments presented here (and in Chapter V) do not attempt to characterize the dopaminergic regulation of cortically-projecting cholinergic neurons according to this

new classification of DA receptors. Therefore, for the purposes of this thesis, "D_1" and "D_2" should be interpreted as D_1-like and D_2-like, respectively.

The results of the pharmacological experiments presented here, using selective agonists and antagonists for D_1 and D_2 type dopamine receptors, suggest that DA regulates the activity of cortically-projecting cholinergic neurons primarily *via* actions at D_1 receptors. Thus, the selective D_1 agonist significantly increased cortical ACh release while the D_2 agonists did not. In addition, the increased cortical ACh release induced by AMPH or apomorphine was blocked to a greater degree by the D_1 antagonist SCH 23390 than by the D_2 antagonists, and only the D_1 antagonist significantly decreased baseline concentrations of ACh.

There is indirect evidence from other sources suggesting that D_1 receptors are of greater importance than D_2 receptors in regulating the activity of cortically-projecting cholinergic neurons. Measures which are at least in part cholinergically mediated, such as behavioural hyperactivity, EEG desynchrony, and reversal of narcosis, are affected more by D_1 receptor stimulation and blockade (Bagetta *et al.*, 1987; Ongini *et al.*, 1985) than by D_2 manipulations (Horita and Carino, 1991; Horita *et al.*, 1991; Ongini and Longo, 1989). In addition, with regard to the possible anatomical sites of dopaminergic regulation of cortical ACh release discussed above, it is noteworthy that D_1 receptors are more numerous than D_2 receptors in the basal forebrain (Zilles *et al.*, 1991), in most regions and laminae of the cortex (Richfield *et al.*, 1989) and in the nucleus accumbens (Richfield *et al.*, 1987). It has also been demonstrated recently that the D_1 agonist used in the present experiments induces expression of Fos-like immunoreactivity, which is often considered a marker of neuronal activity, in cortically-projecting cholinergic neurons in the basal forebrain (Robertson and Staines, 1994). Existing electrophysiological evidence, that a D_1 agonist increases activity while a D_2 agonist decreases activity in a majority of basal forebrain neurons (Maslowski and Napier,

1991), neither supports nor contradicts the data presented here given that the neurons under investigation were not identified as cholinergic or cortically-projecting.

In contrast to the proposition that D_1 receptors are of primary importance for dopaminergic regulation of cortical ACh relese, Casamenti *et al.* (1987) have reported that AMPH-induced increases in cortical ACh release, as assessed by a cortical cup technique in immobilized rats, are blocked by a high dose of haloperidol (1.0 mg/kg) and not by a low dose of SCH 23390 (0.003 mg/kg). These researchers also reported that high doses of quinpirole (1.0 and 2.5 mg/kg) significantly increased cortical ACh release to a peak of 44 and 51%, respectively, over baseline, while the partial D_1 agonist SKF 38393 (1.25 and 10 mg/kg) was without effect. Interestingly, in pilot experiments in this laboratory, SKF 38393 at 20 mg/kg robustly increased cortical ACh to 350% of baseline. The difference between the present results and these earlier observations may be due to the use of different doses, and/or to the use of freely moving animals and more physiologically relevant sampling techniques in the present study.

Although the evidence presented here indicates that D_1 receptors predominate over D_2 receptors in mediating the effects of DA on CBC neurons, several observations suggest that D_2 receptor stimulation may also participate. D_2 antagonists partially blocked the effect of AMPH and apomorphine on cortical ACh release and slightly, but insignificantly, decreased basal dialysate values of cortical ACh. Thus, in attempting to determine the relative contribution of D_2 receptors in regulating cortical ACh release, an inconsistency has become apparent: D_2 receptor antagonists are somewhat effective while D_2 agonists are not. It remains possible that D_2 agonists have minor excitatory actions on the basal forebrain cholinergic system that were not revealed by the present experiments. For example, although the effects of the D_2 agonists were not statistically significant, extracellular ACh concentrations were somewhat increased in the second hour after administration of the drugs. This is in contrast to quinpirole- and PHNO-induced decreases of ACh release in the striatum (Bertorelli and Consolo, 1990;

Robertson *et al.*, 1993; Timmerman and Westerink, 1991), which are not larger in the second hour after administration than in the first. It is unlikely that the lack of significant effect was due to insufficient dosage, because the doses of quinpirole and PHNO used here have been shown by others to have behavioural and/or neurochemical effects (Bertorelli and Consolo, 1990; Martin *et al.*, 1984; Robertson *et al.*, 1993). The present experiments also demonstrated that the expression of an otherwise silent D_2 effect on cortical ACh release is not enabled by D_1 receptor stimulation. Interactions between D_1 and D_2 receptors have been proposed to explain certain other D_2 mediated effects (Clark and White, 1987; Waddington and O'Boyle, 1989). However, quinpirole had no effect when paired with the D_1 agonist in the present experiments, and therefore such interactions do not appear to be important in the regulation of the basal forebrain cholinergic system.

If D_2 receptors are not involved in regulating cortical ACh release, as some of these experiments suggest, it remains to be explained why D_2 antagonists significantly attenuate AMPH-induced increases in cortical ACh release. It is possible that a non-dopaminergic contribution to AMPH's effect on cortical ACh release is blocked by these D_2 antagonists by virtue of their effects on other monoamine receptors (Christensen *et al.*, 1984; Hall *et al.*, 1986; Ogren *et al.*, 1986). In addition, it was demonstrated in Chapter II that there is a significant relationship between locomotor activity and cortical ACh release across a number of conditions. Therefore, because raclopride and haloperidol both reduce AMPH-induced increases in locomotor activity, it is possible that these D_2 receptor antagonists block that portion of AMPH's effects on cortical ACh release that is associated with increases in locomotor activity. It is noteworthy in this regard that the increasing trend of cortical ACh release in the second hour after administration of the D_2 agonists corresponds to the time of increased locomotion reported after injection of 0.5 mg/kg quinpirole (Eilam and Szechtman, 1989).

Given the previously demonstrated correlation between cortical ACh release and behavioural activity, it is interesting that the DA receptor antagonists blocked the AMPH-induced locomotor activity while failing to antagonize completely the AMPH-induced increase in ACh release and that the D_2 agonists stimulated behaviour without increasing cortical ACh release. In addition, unlike more potent D_1 agonists (Acquas *et al.*, 1994) the D_1 agonist used in the present studies did not increase locomotion, although it did increase cortical ACh release. Thus, pharmacological treatments can dissociate the usually positive correlation between ACh release and locomotion. More precise behavioural characterizations might help reveal the basis of the correlation and its dissociation by pharmacological treatments.

The experiments reported here indicate that D_1 receptors primarily mediate the excitatory effects of dopaminergic drugs on cortically-projecting CBC neurons. A minor role of D_2 receptors cannot be excluded, however, and the basis of the discrepancy between the lack of effect of D_2 agonists and the partial effects of D_2 antagonists remains to be determined.

IV. ATTENUATION OF AMPHETAMINE-INDUCED INCREASES OF CORTICAL ACETYLCHOLINE RELEASE BY FOREBRAIN DEPLETIONS OF DOPAMINE, BUT NOT NORADRENALINE

(A) INTRODUCTION

As discussed previously, cholinergic projections to the neocortex which arise from the basal forebrain (Lehmann *et al.*, 1980) are critically involved in cortical activation (Semba, 1991). The stimulant drug AMPH desynchronizes the cortical EEG, causes behavioural hyperactivity (Fairchild *et al.*, 1967), and increases the activity of neurons in the CBC as measured by increased cortical ACh turnover (Robinson *et al.*, 1978) and release (Chapter III; Casamenti *et al.*, 1986; 1987; Hemsworth and Neal, 1968; Okada, 1991; Pepeu and Bartolini, 1968). These effects of AMPH are likely mediated indirectly by DA and/or NA, the neuronal release of which are increased by this drug (Azzaro and Rutledge, 1973; Kuczenski and Segal, 1989). In support of this hypothesis, dopaminergic and noradrenergic neurons innervate the cortex (Fuxe *et al.*, 1968; Lindvall *et al.*, 1974) and the region in the basal forebrain containing cholinergic perikarya (Semba *et al.*, 1988; Zaborszky *et al.*, 1991). Receptors for DA and NA are also present in both the cortex (Palacios and Kuhar, 1980; Richfield *et al.*, 1989; Sargent Jones *et al.*, 1985) and basal forebrain (Zilles *et al.*, 1991). Multi-synaptic pathways may also account for the effect of DA and/or NA on cortical ACh release. Of these possible sites of interaction, the cortex has been ruled out given the lack of effect of cortically-applied AMPH on cortical ACh release (Chapter III; Beani and Bianchi, 1973; Pepeu and Bartolini, 1968).

Pharmacological experiments have also suggested an interaction of both DA and NA with CBC neurons. Nonselective DA- and D_1- receptor agonists increase cortical ACh release and D_1 receptor antagonists decrease basal release and block AMPH-induced release of ACh in the cortex (Chapter III; Mantovani *et al.*, 1977). In contrast,

although effects of noradrenergic agents on cortical ACh release have been reported, these seem unable to account for AMPH-induced increases in cortical ACh release. Thus, application of NA or stimulation of noradrenergic cell bodies in the locus coeruleus decreases cortical ACh release, apparently *via* actions on alpha-noradrenergic receptors (Beani *et al.*, 1978; Bianchi *et al.*, 1979; Vizi, 1980). Conversely, while alpha receptor antagonists have been reported to block AMPH-induced increases in cortical ACh turnover (Robinson *et al.*, 1978), Bartolini and Pepeu (1970) found that beta, but not alpha, blockers attenuated the effect of AMPH on cortical ACh release. More recently, it has been reported that NA depolarizes identified CBC neurons in guinea pig brain slices (Fort *et al.*, 1993). An understanding of these apparently contradictory results awaits further study using the more selective drugs and physiological techniques that are now available.

The present experiments further assessed the extent to which forebrain DA and/or NA mediate AMPH-induced stimulation of CBC neurons. To this end, AMPH-induced changes in cortical microdialysate concentrations of ACh were measured in animals depleted of forebrain DA or NA by neurotoxic lesions. In addition, the effect on cortical ACh release of an arousing non-pharmacological stimulus, injection of vehicle (Chapter II), was examined in rats depleted of NA to test for a possible interaction of locus coeruleus NA neurons and CBC neurons in cortical arousal (Berridge and Foote, 1991; Foote *et al.*, 1980; Semba, 1991).

(B) MATERIALS AND METHODS

Experimental protocol and drugs

Male Wistar rats (270-310 g) received unilateral 6-hydroxydopamine (6-OHDA) lesions of the mesotelencephalic dopamine system (MDS), bilateral 6-OHDA lesions of

the dorsal noradrenergic bundle (DNB), or control surgeries and were group housed for 2 weeks with food and water available *ad libitum*. Following surgical implantation of a microdialysis probe into the frontal cortex at the end of this 2 week period, all rats were housed individually in Plexiglas cages (35x35x25 cm) and maintained on a 12:12 h light:dark schedule with food and water available *ad libitum*. Dialysis was initiated two days after probe implantation, during the rats' normal "lights on" phase, and experiments involved the subcutaneous injection of vehicle or AMPH (2.0 mg/kg; BDH). This dose of AMPH has significant behavioural effects and reliably increases ACh release in the frontal cortex (Chapter III). An unlesioned group of rats with bilateral frontal cortex probes was also dialyzed with perfusates containing the sodium channel blocker TTX (1*u*M) or lacking calcium ions.

Unilateral 6-OHDA lesions of the mesotelencephalic DA system

Although theoretically preferable, bilateral lesions of the MDS were precluded by ensuing aphagia and adipsia in bilaterally lesioned animals (Fibiger *et al.*, 1973). Therefore, unilateral lesions were performed as follows: rats were pretreated with desmethylimipramine (20 mg/kg intraperitoneal (i.p.)) 30 min prior to surgery, anaesthetized with sodium pentobarbital (50-60 mg/kg i.p.), and placed in a stereotaxic frame with the incisor bar positioned 4.2 mm below interaural zero. A 30 gauge stainless steel cannula was lowered through a burr hole in the skull to a site in the lateral hypothalamus containing axons of the MDS (A:+5.9, L:+2.3, D:+2.2 measured from interaural zero). The cannula was attached by polyethylene tubing to a 5 *u*l Hamilton syringe driven by a syringe pump (Harvard Apparatus), all shielded from light. 6-OHDA HBr (3.0 mg/ml, Sigma) was dissolved in a vehicle consisting of 0.5 mg/ml ascorbic acid in 0.9% saline and kept on ice in the dark before use. Following a two minute delay after the cannula was positioned in the target site, the 6-OHDA solution was infused at 0.2 *u*l/min over 20 min to yield a dose of 8 *u*g base/4 *u*l/site. After a

further 5 min to allow for diffusion, the cannula was removed and the wound stitched closed. Control animals were pretreated identically to the lesioned animals; the cannula was aimed at the same co-ordinates, but lowered only into the cortex (D:+8.0 from interaural zero) and left there for 5 min.

Bilateral 6-OHDA lesions of the dorsal noradrenergic bundle

Rats were anaesthetized with sodium pentobarbital (50-60 mg/kg i.p.) and placed in a stereotaxic frame with the incisor bar 3.3 mm below interaural zero. Simultaneous bilateral injections of 6-OHDA were carried out as described above with the following differences: the cannulae were positioned in the DNB (A:+2.6, L:$\pm$1.0, D:+3.6 measured from interaural zero), and 6-OHDA (2.0 mg/ml) was infused at 0.2 *u*l/min over 10 min to yield a dose of 2.7 *u*g base/2 *u*l/side. For the surgical control group, the cannulae were aimed at the same anterior/lateral co-ordinates, but lowered only into the cortex (D:+8.6 from interaural zero) and left there for 5 min.

Probe implantation and microdialysis

Brain microdialysis was performed as in Chapter III with the following exceptions. The probes were made of acrylonitrile-sodium methallyl sulfonate fiber (inner diameter = 220 *u*m, outer diameter = 310 *u*m, molecular weight cut off > 60 000 Dalton; Filtral AN69, Hospal) and had an active surface length of 10.9 mm in the DNB lesioned and control groups or 5.45 mm in the MDS lesioned and control groups. In the latter groups, only the half of the probe situated in the cortical hemisphere ipsilateral to the lesion was left unblocked and thus active for dialysis.

To prevent twisting of inlet and outlet tubing due to excessive turning in the MDS lesioned rats treated with AMPH, the tubings of these rats and their controls were attached to a liquid swivel which was anchored to a "vest" worn by the rat.

In vitro recovery of probes

Unused bilateral cortical microdialysis probes were perfused with the neostigmine-containing perfusion solution while immersed in a standard solution of ACh/Ch maintained at 37^{o}C. The recovery of ACh across the membrane was calculated as a percentage of the standard solution.

Assay of ACh

ACh was assayed by HPLC-ECD as described in Chapter II, with the following additions to the technical equipment: an HPLC pump (Shimadzu) and a platinum electrode/reference electrode assembly (Antec).

Tissue level analysis

To determine the efficacy of the neurotoxic lesions, representative dopaminergic terminal regions of the MDS (striatum and nucleus accumbens (NAc)) and representative noradrenergic terminal regions of the DNB (frontal cortex (FCTX), parietal cortex (PCTX) and hippocampus) were assayed for biogenic amine content. Rats were killed by cervical dislocation and the brains were rapidly removed. To assess DA depletion in the MDS lesioned rats, 2 coronal sections of forebrain were cut with a freezing microtome, spanning the following anterior coordinates (in mm) measured from bregma (from the atlas of Paxinos and Watson, 1986): 1.2-2.2 for NAc and 0.2-1.2 for striatum. To assess NA depletion in the DNB lesioned rats, 2 coronal sections were cut using a McIlwain tissue chopper, spanning 1.2-5.2 for the FCTX and (-1.8)-(+0.2) for the PCTX. From the remaining caudal portion of brain, the hippocampi were hand-dissected. The brain sections were placed on ice-cold, dampened filter paper where DA and NA terminal regions were hand-dissected from both hemispheres with the aid of binocular magnifiers. This procedure yielded unilateral tissue samples having mean weights of 9.6 mg for NAc, 13.2 mg for striatum, 26.4 mg for FCTX, 18.6 mg for

PCTX and 17.0 mg for hippocampus. Tissue samples were sonicated in ice-cold 0.2 M $HClO_4$ containing 0.15% $Na_2S_2O_3$ and 0.05% Na_2EDTA (NAc, FCTX and PCTX in 300 *u*l, striatum in 400 *u*l, hippocampus in 500 *u*l). After centrifugation, samples were stored at -80°C until the time of their assay. Thawed samples were injected from a 100 *u*l loop on a valve (Valco) onto a reverse phase analytical column (nucleosil 5 *u*m C18; Phenomenex). The mobile phase consisted of 0.1 M sodium acetate buffer, 0.01 mM Na_2EDTA, 0.75-0.85 mM octane sulfonic acid, 10-12% methanol, pH 3.6 and was delivered at 0.35-0.8 ml/min by a pump (PM-48, BAS or Shimadzu LC-600). Solutes were quantified by electrochemical detection using an LC-4B amperometric detector (BAS) with a glassy carbon working electrode at +0.7 V *vs.* a Ag/AgCl reference electrode. The detection limit for all compounds under these conditions was between 10 and 100 fmol/injection.

Retrograde labelling

Unilateral cortical probes, in MDS lesioned and control rats which could not be used for dialysis experiments due to technical problems (n=3), were filled with a 4% solution of Fluoro-Gold (Fluorochrome, Inc.) in 0.9% saline, and flushed with saline 12 h later. One week later these animals were sacrificed and coronal forebrain slices were collected, processed and examined under ultraviolet light as described by Robertson *et al.* (1991).

Statistical analyses

Microdialysate outputs in each animal were calculated as a percent of baseline, 100% baseline being defined as the average of the last six pre-drug concentrations (fmol/min). As an alternate method of correcting for inter-animal variability in baseline output, data from individual animals were also transformed into "delta fmol/min" by subtracting the average of the last six pre-drug concentrations (fmol/min) from the

absolute concentration (fmol/min) of each dialysate sample. Univariate ANOVAs were used to compare basal microdialysate outputs (fmol/min) of cortical ACh in lesion *vs.* control groups for the two lesion types. ANOVAs with repeated measures were used to compare treatment-induced changes in cortical microdialysate outputs of ACh (in both % baseline and delta fmol/min scores) in surgical control *vs.* 6-OHDA lesioned rats, and the main treatment effect is reported. Treatment x time interaction effects are also reported which include Huynh-Feldt adjustments of the degrees of freedom to account for the use of time as a repeated measure. One-way analyses of variance with Tukey-"highly significant differences" post-hoc comparisons were used to assess differences in lesion-induced tissue concentrations of monoamines.

(C) RESULTS

Efficacy of lesions

Table 3 shows the effects of 6-OHDA lesions of the MDS on tissue concentrations of DA, NA and 5-HT. DA was significantly depleted in the striatum and NAc ipsilateral to the lesioned MDS, and decreased to a lesser extent in the contralateral NAc. NA concentrations were also significantly reduced ipsilaterally by these lesions. Tissue concentrations of 5-HT were not affected in any region.

The results of 6-OHDA DNB lesions on regional tissue concentrations of DA, NA and 5-HT are summarized in Table 4. NA was significantly depleted in all 3 regions, leaving tissue concentrations of DA and 5-HT unaffected.

Site of neurons projecting to unilateral cortical probe

Application of Fluoro-Gold through the unilateral cortical probe retrogradely labeled cell bodies in, among other areas, the basal forebrain (data not shown). While

Table 3. Effects of 6-OHDA lesions of the mesotelencephalic dopamine system on regional tissue concentrations of dopamine, noradrenaline, and serotonin

	Tissue concentration pmol/mg tissue ± S.E.M. (% surgical control)		
Region	Surgical Control	Lesion Control[a]	Lesion
DOPAMINE			
N. accumbens	39.1±2.8	27.9±1.5 (71.4%)#	0.19±0.06 (0.49%)*
Striatum	57.6±3.3	57.6±2.5 (100.0%)	0.06±0.02 (0.10%)*
NORADRENALINE			
N. accumbens	0.96±0.09	0.71±0.08 (74.0%)	0.36±0.07 (37.5%)*
Striatum	0.48±0.05	0.62±0.07 (129.2%)	0.11±0.05 (22.9%)*
SEROTONIN			
N. accumbens	2.68±0.26	2.24±0.15 (83.7%)	2.59±0.26 (96.8%)
Striatum	1.23±0.08	1.08±0.06 (87.6%)	1.04±0.13 (84.3%)

Tissue was assayed from 6 surgical control rats and 10 6-OHDA lesioned rats.

[a]Lesion control samples are from the hemisphere contralateral to the lesion.
*Values are significantly different from both the surgical control and the lesion control, $p<0.05$.
#Value is significantly different from both the surgical control and the lesion, $p<0.05$.

Table 4. Effects of 6-OHDA lesions of the dorsal noradrenergic bundle on regional tissue concentrations of dopamine, noradrenaline, and serotonin

Region	Tissue concentration pmol/mg tissue ± S.E.M. (% surgical control) Surgical Control	Lesion
DOPAMINE		
Frontal Cortex	0.33±0.02	0.34±0.03 (102.6%)
Parietal Cortex	0.11±0.04	0.076±0.013 (70.2%)
Hippocampus	0.047±0.010	0.037±0.005 (78.5%)
NORADRENALINE		
Frontal Cortex	1.44±0.09	0.048±0.009 (3.3%)*
Parietal Cortex	1.07±0.06	0.022±0.005 (2.1%)*
Hippocampus	1.85±0.14	0.10±0.03 (5.6%)*
SEROTONIN		
Frontal Cortex	2.85±0.17	3.05±0.24 (106.7%)
Parietal Cortex	1.19±0.09	1.41±0.11 (118.5%)
Hippocampus	1.87±0.13	2.10±0.16 (111.9%)

Tissue was assayed from 7 surgical control rats and 9 6-OHDA lesioned rats, with the exception of basal forebrain tissue which was dissected from 5 surgical control rats and 7 6-OHDA lesioned rats.

*Values are significantly different from the surgical control, $p<0.05$.

the vast majority of the neurons labeled in this region were in the hemisphere ipsilateral to the open side of the probe, a few labeled neurons could be observed in the contralateral basal forebrain.

Characterization of probes made with acrylonitrile fiber

The effect of locally-perfused TTX (1 *u*M) on cortical ACh output recovered by the acrylonitrile membrane is shown in Fig. 13. Dialysate concentrations of ACh decreased significantly during the 60 min of TTX perfusion [$F(1.06,4.24)=11.99$, $p=0.023$]. Calcium-free perfusion also reduced ACh output by 75-80% in two rats (data not shown). The unlesioned rats used for these experiments had an average basal output ($\pm$ S.E.M.) of 68.34 $\pm$ 10.79 fmol/min (n=7). *In vitro* recovery of the bilateral cortex probe, using Hospal membrane, was 51.7 $\pm$ 2.5% (n=3).

Effects of AMPH on cortical ACh release in DA- or NA-depleted rats

The average outputs of ACh from the unilateral cortical probes in the MDS lesioned and control groups (in fmol/min $\pm$ S.E.M.) were 44.1 $\pm$ 4.9 and 31.6 $\pm$ 7.6 respectively. The bilateral cortical probes yielded average outputs of 67.8 $\pm$ 10.3 in DNB lesioned rats, and 85.9 $\pm$ 13.4 in their surgical controls. These values were not significantly different in the MDS groups [$F(1,12)=2.25$, $p=0.16$] or the DNB groups [$F(1,14)=1.19$, $p=0.29$].

The effects of AMPH on ACh release in the cortex of DA-depleted rats and their surgical controls are shown in Fig. 14. In surgical control animals, AMPH increased ACh release to a maximum of approximately 270% of baseline (Fig. 14A) or by 40 fmol/min (Fig. 14B), for 3 h. In contrast, ACh release increased to 160% of baseline, or by 25 fmol/min in the 6-OHDA MDS-lesioned rats. The AMPH-induced increases in ACh release in the cortex of these two groups of animals differed significantly, using data calculated as percent baseline [$F(1,12)=8.92$, $p=0.011$]. The more conservative

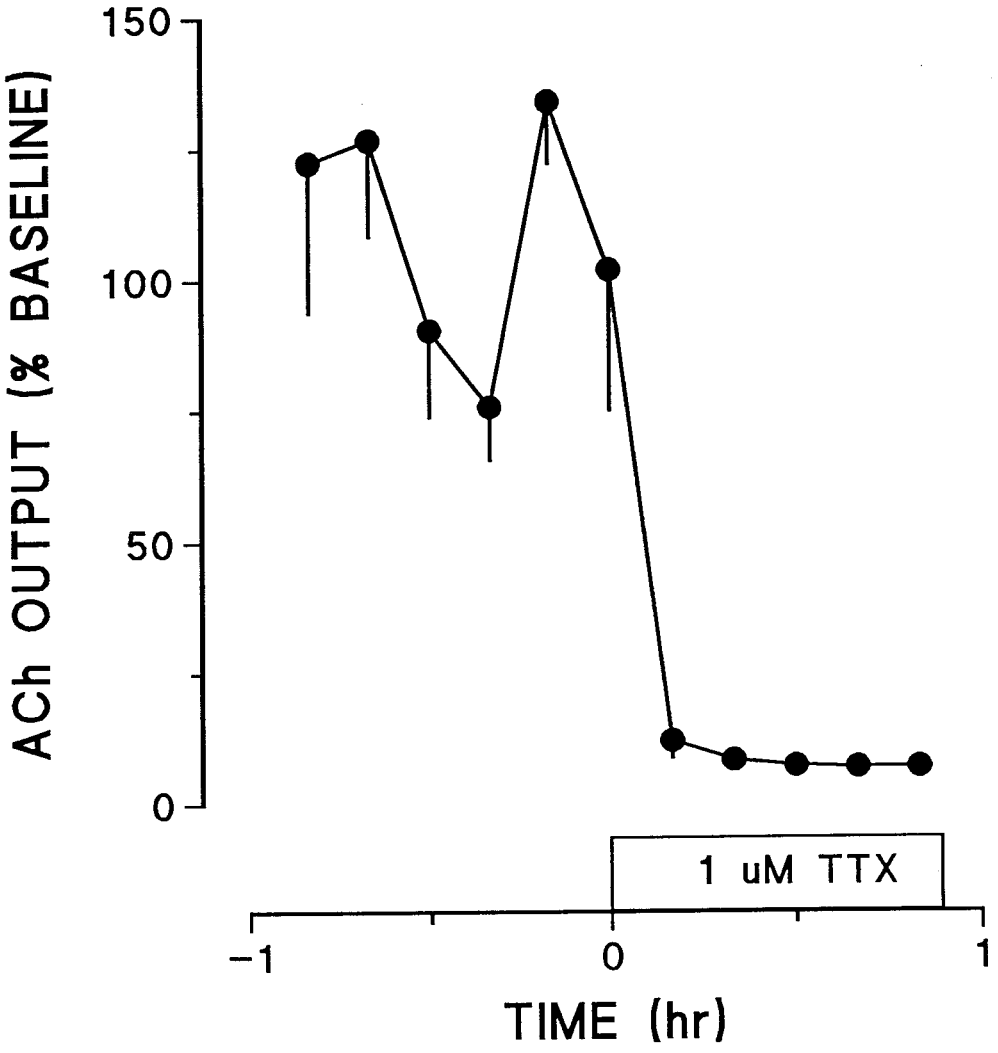


Figure 13. Tetrodotoxin (TTX)-sensitivity of cortical ACh recovered by a transverse dialysis probe made of acrylonitrile fibre. TTX (1 *u*M) was included in the perfusate for 1 h. Data points represent group means (n=5) ± S.E.M.

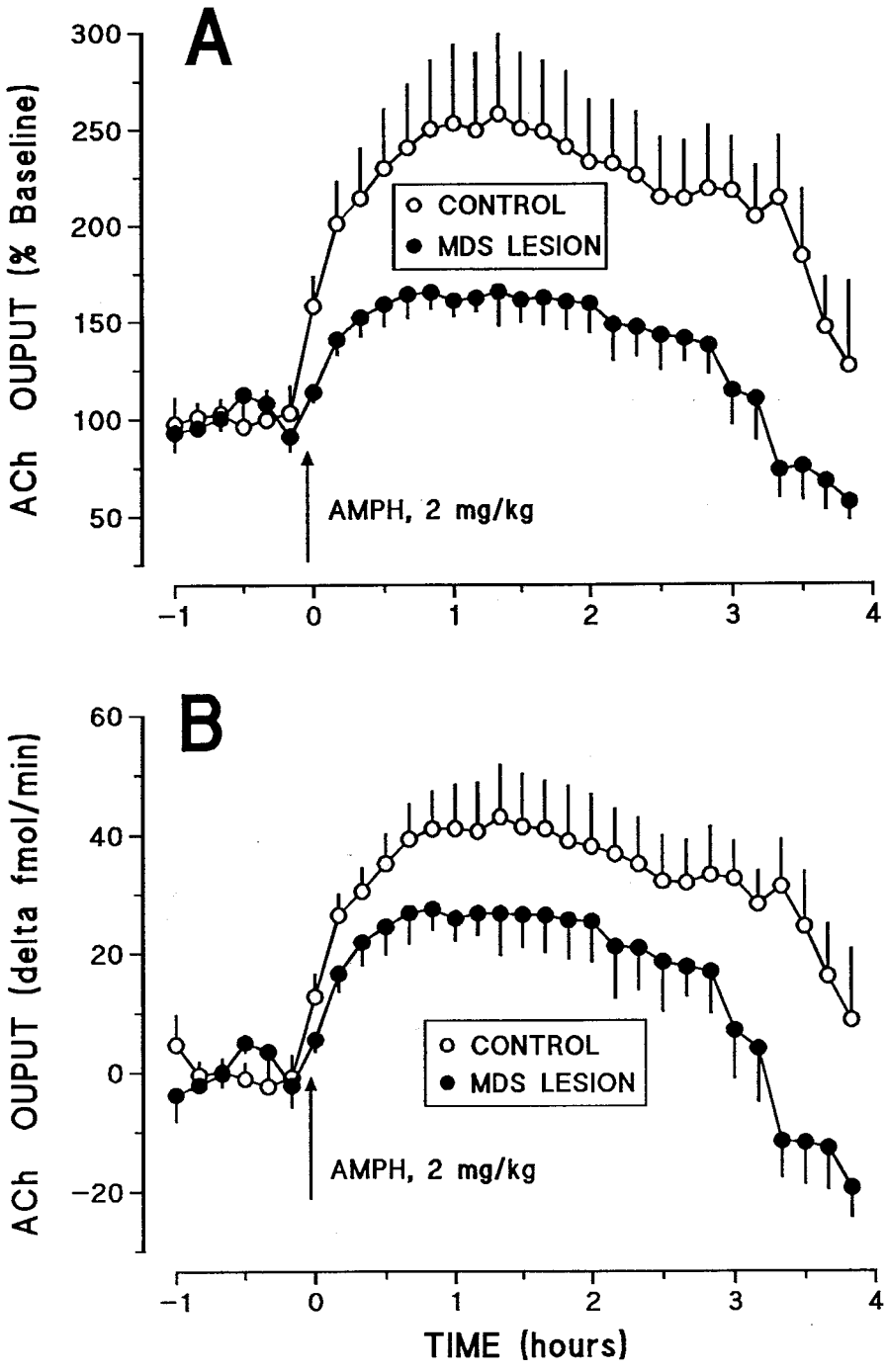


Figure 14. Dialysate values of ACh in the frontal cortex of rats having unilateral 6-OHDA lesions of the mesotelencephalic dopamine system (MDS; closed circles, n=9) or of control rats (open circles, n=5) after injection of *d*-amphetamine (AMPH; 2.0 mg/kg). Data points represent group means ± S.E.M. and are presented as A: percentage of baseline, or B: delta fmol/min (see Experimental Procedures).

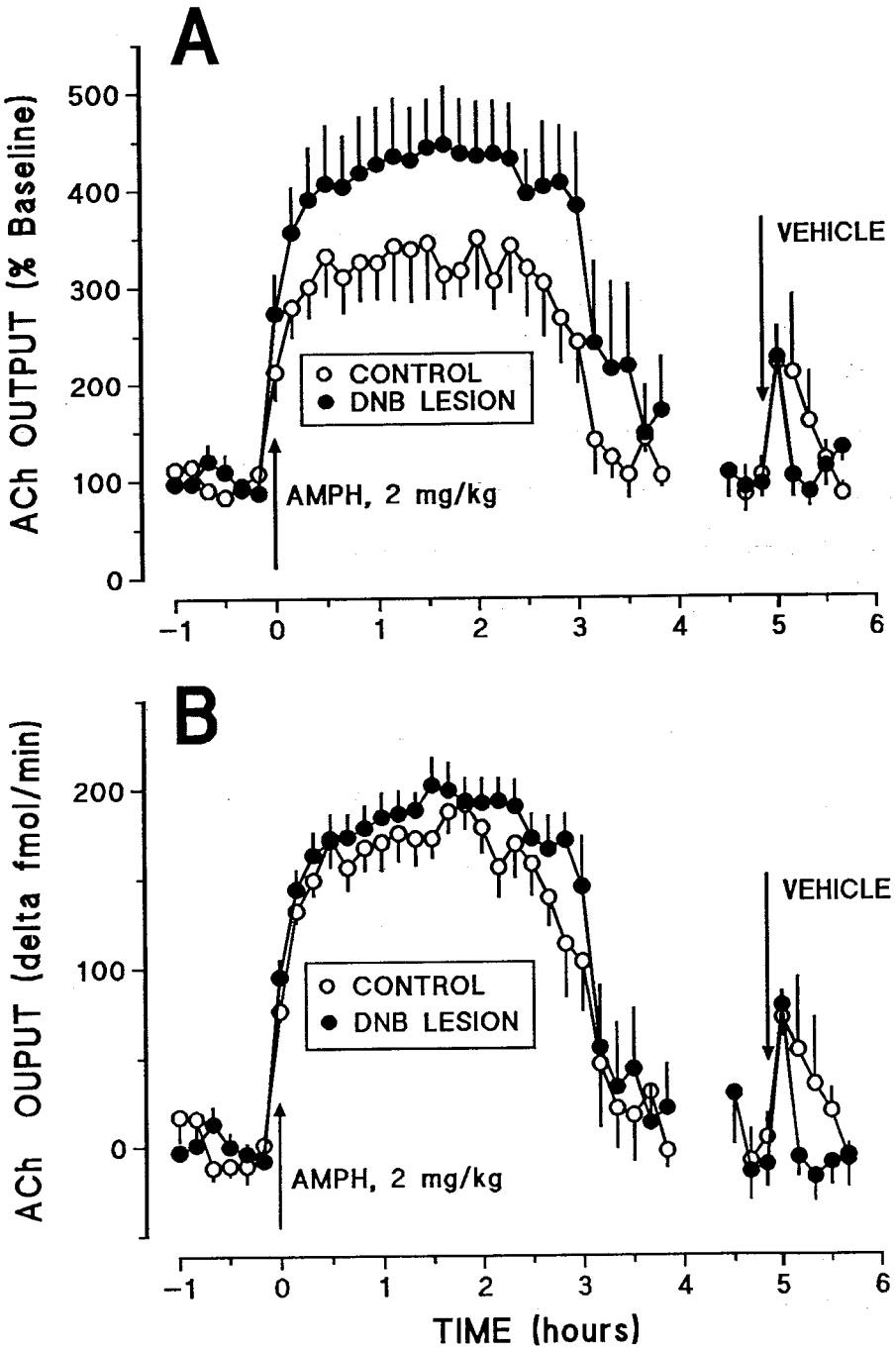


Figure 15. Dialysate values of ACh in the frontal cortex of rats having bilateral 6-OHDA lesions of the dorsal noradrenergic bundle (DNB; closed circles) or of control rats (open circles) after injection of *d*-amphetamine (AMPH; 2.0 mg/kg; DNB lesion n=9, control n=7) and vehicle (1.0 ml/kg; DNB lesion n=4-7, control n=3-5). Data points represent group means ± S.E.M. and are presented as A: percentage of baseline, or B: delta fmol/min (see Experimental Procedures).

treatment of data, using delta fmol/min, yielded a main effect approaching significance at the 5% level [$F(1,12)=4.21$, $p=0.063$] and a significant treatment x time interaction [$F(8.74,104.87)=2.54$, $p=0.012$]. Following AMPH administration, both groups of rats were behaviourally hyperactive and the 6-OHDA lesioned rats rotated ipsilaterally.

Fig. 15 illustrates the effects of AMPH and vehicle administration in NA-depleted rats and their surgical controls. When calculated as percent baseline (Fig. 15A), ACh release in the cortex was increased by AMPH to a maximum of 350% for 3 h in the control group and to a greater extent (430% of baseline) in the 6-OHDA DNB-lesioned group. The effect of AMPH was not significantly different between these two groups [$F(1,14)=1.89$, $p=0.191$]. This result is more evident when the data are represented as delta fmol/min (Fig. 15B). AMPH increased ACh release by 200 fmol/min in both lesioned and control animals, with no significant difference between these groups [$F(1,14)=1.19$, $p=0.294$]. The effect of vehicle injections on cortical ACh release, a transient increase, was not significantly different between the NA-depleted and control animals using either form of data representation [$F(1,10)=1.73$, $p=0.218$ for % baseline data and $F(1,10)=1.46$, $p=0.255$ for delta fmol/min].

(D) DISCUSSION

The results reported here point to a more important role of forebrain DA than NA in regulating cortically-projecting cholinergic neurons, given that 6-OHDA lesions of the MDS attenuated AMPH-induced increases in cortical ACh release, whereas 6-OHDA lesions of the DNB did not. The increase in cortical ACh release caused by an arousing stimulus was also unaffected by the forebrain NA depletion produced by the DNB lesion. The finding that DA mechanisms contribute significantly to AMPH's effects on cortical ACh release supports the pharmacological experiments described in Chapter III.

Technical considerations

Dialysate concentrations of cortical ACh were found to be calcium-dependent and TTX-sensitive using probes made of acrylonitrile membrane. Although the *in vitro* recovery of this membrane (52%) is superior to that of the cellulose ester fiber reported in Chapter II (33%), the average basal output found here using the acrylonitrile fiber in the cortex of unlesioned rats (68 ± 11 fmol/min) is only somewhat higher than that found in Chapter III using cellulose ester fiber (47 ± 7 fmol/min). This finding is not unreasonable in view of the theory that *in vivo* factors, rather than *in vitro* recoveries, are the limitting factors determining the recoveries of dialysis probes (Benveniste *et al.*, 1989). The present results demonstrate that the acrylonitrile membrane is a suitable alternative for the construction of transverse microdialysis probes for recovery of ACh.

An unexpected complication in the present experiments was that the 6-OHDA lesion of the MDS with desmethylimipramine pretreatment did not selectively deplete ipsilateral DA. The contralateral NAc showed a small but significant depletion of DA, and NA was significantly depleted in both the striatum and NAc. However, because the more substantial NA depletion after DNB lesions did not affect AMPH-induced increases in cortical ACh release, it can be concluded that the attenuation of AMPH's effects by the MDS lesion was not noradrenergically mediated.

Technical factors may also have contributed to an underestimation of the effect of unilateral MDS lesions on AMPH-induced cortical ACh release. Because Fluoro-Gold administered through the unilateral probe retrogradely labelled a small number of neurons in the contralateral basal forebrain, it is possible that this molecule diffused from the open side of the probe to the cortex contralateral to the lesion; similarly, a small amount of ACh released from the contralateral cortex may have been recovered by the unilateral probe. Alternatively, contralateral Fluoro-Gold staining in the basal forebrain could be caused by crossed cholinergic projections; however, this possibility

has received no previous neuroanatomical support. Either possibility would account for a small portion of ACh in the dialysate being unaffected by the unilateral MDS lesion.

A final technical consideration requires discussion. Because the differences in basal output between lesioned *vs.* control groups were large but not statistically significant, an alternate method for presenting the dialysis data was developed. Unlike the percent baseline calculations, the delta fmol/min measure removes the potentially misleading effects that differences in average basal output can have on percent scores. This is well illustrated in Fig. 15: when presented in the conventional percent baseline format, the DNB lesions appear to have potentiated ACh release compared to the control group. It is obvious, however, using the delta fmol/min measure (Fig. 15B), that there was no difference between the groups in AMPH-stimulated ACh release, and the apparent difference seen in Fig. 15A was entirely due to the non-significant baseline differences between the two groups. In addition, the error terms are reduced using the latter measure. This example demonstrates that where moderate differences in basal output between experimental groups exist, conventional percent baseline data presentation can yield potentially misleading results. This problem can be overcome using the delta fmol/min calculation.

Regulation of cortical ACh release by monoamines

The data reported here confirm that DA regulates cortically-projecting cholinergic neurons. In support of this conclusion, nonselective DA- and selective D_1- receptor agonists increase cortical ACh release (Chapter III; Casamenti *et al.*, 1986; Mantovani *et al.*, 1977). In addition, it has recently been demonstrated that a population of cortically-projecting CBC neurons are stimulated by a D_1 agonist, assessed using the expression of Fos-like immunoreactivity as a marker of neuronal activity; this same population of neurons exhibits markedly reduced ChAT immunoreactivity after 6-OHDA lesions of the MDS (Robertson and Staines, 1994). These authors thus speculate that destruction of DA

neurons may have deprived CBC neurons of an excitatory drive. As outlined in the Introduction, previous evidence of noradrenergic regulation of CBC neurons is inconsistent, given that both excitatory (Bartolini and Pepeu, 1970; Fort *et al.*, 1993; Robinson *et al.*, 1978) and inhibitory (Beani *et al.*, 1978; Bianchi *et al.*, 1979; Vizi, 1980) interactions have been reported.

Previous experiments using rats depleted of monoamines further support the conclusion that DA, but not NA, mediates the effect of AMPH on basal forebrain cholinergic neurons. Casamenti *et al.* reported that presumed depletions of DA (1986) but not NA (1987) blocked AMPH-induced increases of cortical ACh overflow measured using the cortical cup technique. In addition, Nilsson *et al.* (1992) reported that DA, but not NA, depletions attenuate AMPH-induced increases of hippocampal microdialysate concentrations of ACh.

It is perhaps surprising that neither of the two lesion conditions produced larger effects on AMPH-induced cortical ACh release. AMPH is not entirely selective for the release of NA and DA, and also increases synaptic concentrations of serotonin (5-HT) (Azzaro and Rutledge, 1973; Kuczenski and Segal, 1989). Because 5-HT also appears to stimulate cortical ACh release (Hirano, Day, and Fibiger, submitted), a serotonergic contribution to the effect of AMPH on cortical ACh release cannot be excluded. This possibility should be explored further. It should be noted, however, that it is not supported by previous cortical cup data which showed no effect of the 5-HT synthesis inhibitor, para-chlorophenylalanine, on the AMPH-induced increase in ACh release (Casamenti *et al.*, 1987).

One possible explanation for the failure of the extensive unilateral MDS lesions to more effectively attenuate AMPH-induced cortical ACh release is that AMPH-induced DA release in the intact hemisphere may have mediated the lesion resistant effect of the drug on cortical ACh release. The precise neuroanatomical systems mediating this effect of AMPH have not been delineated and may include crossed projections. This

possibility is supported by the fact that systemic pharmacological blockade of AMPH-induced cortical ACh release by the D_1 receptor antagonist SCH 23390 (Chapter III) is greater than that produced by the unilateral DA lesions used here.

Although the experiments presented here provide no evidence of noradrenergic regulation of cortically-projecting cholinergic neurons, it remains possible that NA does affect this system but that the DNB lesions left the relevant NA projection intact. Unlike the MDS, which accounts for almost all DA in the forebrain, the noradrenergic innervation consists of a dorsal tract (DNB) and a ventral tract (VNB) (Ungerstedt, 1971). Thus, before excluding NA as a regulator of cortically-projecting cholinergic neurons, it will be necessary to determine if VNB lesions can attenuate AMPH-induced cortical ACh release. In this regard, it is noteworthy that AMPH-induced increases in hippocampal ACh release is not affected by VNB lesions (Nilsson *et al.*, 1992).

V. DOPAMINERGIC REGULATION OF HIPPOCAMPAL ACETYCHOLINE RELEASE

(A) INTRODUCTION

Cholinergic projections to the hippocampus originate from perikarya in the medial septum and vertical limb of the diagonal band of Broca (Fibiger, 1982). Rather than being arranged as discrete nuclei these cholinergic neurons, along with cholinergic neurons projecting to the cerebral cortex, appear to form an anatomical continuum in the basal forebrain (Schwaber *et al.*, 1987). Given this anatomical organization, the CBC may to some extent operate as a single functional unit. According to this hypothesis, measures of cholinergic neuronal activity in the hippocampus and cortex should be similar under most circumstances. This hypothesis can be evaluated by examining the responses of these spatially dispersed neurons to pharmacological and physiological challenges.

The hypothesis that the CBC acts as a functional unit is supported by evidence of similar behavioural and pharmacological regulation of cholinergic neurons that project to the cortex and hippocampus. For example, cholinergic activity in both the cortex and hippocampus is increased during arousal, defined behaviourally or electroencephalographically (Chapter II; Kanai and Szerb, 1965; Nilsson *et al.*, 1990), with greater ACh release occurring during waking and paradoxical sleep than during slow wave sleep (Jasper and Tessier, 1971; Kametani and Kawamura, 1990). Furthermore, thyrotropin releasing hormone increases ACh release to a similar extent in the two regions (Giovannini *et al.*, 1991). ACh release in the cortex and hippocampus is also similarly affected by alterations in serotonergic tone (Bertorelli *et al.*, 1992), although serotonin's effects may be mediated by different receptor subtypes in the two regions (Barnes *et al.*, 1989; Maura and Raiteri, 1986). Benzodiazepine agonists decrease

ACh release in the hippocampus (Imperato *et al.*, 1993a); similarly, benzodiazepine inverse agonists increase ACh release in the cortex (Moore *et al.*, 1992). However, it has also been reported that the effects of benzodiazepine inverse agonists on high-affinity choline uptake are different for the hippocampus and cortex (Miller and Chmielewski, 1990). Additional pharmacological evidence suggests that the trans-synaptic regulation of hippocampal and cortical cholinergic activity, although often similar, can be differentiated: local opiate-induced inhibition of ACh release in these two regions is mediated by different receptor subtypes (Lapchak *et al.*, 1989), adenosine is less effective in decreasing ACh release from cortical than hippocampal synaptosomes (Pedata *et al.*, 1986), and not all nootropics appear to affect both cortical and hippocampal cholinergic measures (Pepeu and Spignoli, 1989). Thus, although cholinergic neurons projecting to the hippocampus and cortex respond similarly during the physiological situations examined to date, it remains unclear if these neurons act as a functional continuum, with identical regulation.

The regulation of cortically-projecting basal forebrain cholinergic neurons by DA, acting primarily at receptors of the D_1 subtype, has been described previously (Chapter III, IV). The hypothesis that the CBC acts as a functional unit predicts that hippocampally-projecting cholinergic neurons would be similarly regulated by DA. To test this hypothesis, *in vivo* microdialysate concentrations of ACh in the hippocampus of conscious, freely moving rats were measured after systemic administration of the non-selective DA receptor agonist apomorphine, the D_1-type receptor agonist CY 208-243, and the D_2-type receptor agonist quinpirole. The catecholamine-releasing agent AMPH was administered both locally through the dialysis probe and systemically; the extent to which the D_1 and D_2 receptor antagonists (SCH 23390 and raclopride, respectively) attenuate the AMPH-induced increase in hippocampal ACh release was also investigated.

(B) MATERIALS AND METHODS

Experimental protocol and drugs

Dialysis experiments and drug treatments were as described in Chapter III, including the size and strain of subject rats. For some experiments (see below), rats receiving two vehicle injections separated by 30 min were used as a control group to permit appropriate comparison with each drug treatment.

Surgery and microdialysis

Brain microdialysis was performed as previously described (Chapter III). Transverse dialysis probes were stereotaxically implanted into the hippocampi of rats (A:-4.3, V:-3.3 measured from bregma, according to the atlas of Paxinos and Watson, 1986). The dialysis probes were made of acrylonitrile-sodium methallyl sulfonate fibre (see Chapter IV), with the following exceptions: for the apomorphine and local AMPH experiments, rats were implanted with saponified cellulose ester dialysis membrane (see Chapter II). All probes had an active surface length of 6.8 mm.

Assay of ACh

ACh was assayed by HPLC-ECD as described in Chapter IV.

Statistical analyses

Data were analyzed as described in Chapter III.

(C) RESULTS

The average baseline output of ACh (± S.E.M.) from the hippocampi in all of the animals implanted with acrylonitrile dialysis fibres was 28.77 ± 1.69 fmol/min (n=43). In animals implanted with cellulose ester fibre, the average basal output was 28.60 ± 2.95 fmol/min (n=11). There was no difference in the baseline output between the experimental groups having acrylonitrile probes [$F(7,35)=0.81$], or between those having cellulose ester probes [$F(1,9)=1.46$].

The effects of the non-selective DA receptor agonist apomorphine on hippocampal dialysate output of ACh are shown in Fig. 16. Apomorphine (1.0 mg/kg) increased ACh release, to a maximum of 200% of baseline values, for approximately 90 min. This effect was significant compared to the transient increase measured after vehicle injection [$F(1,8)=7.00$, $p=0.029$]. Behavioural hyperactivity was evident for approximately 90 min after apomorphine injection (data not shown).

The effects of specific D_1 and D_2 receptor agonists on microdialysate concentrations of ACh in the hippocampus are shown in Fig. 17. The specific D_1 agonist CY 208-243 (1 mg/kg) increased hippocampal ACh release for about 2 h, to a maximum of 230%; this effect was significant compared to vehicle injections [$F(1,8)=10.81$, $p=0.011$]. In contrast, the specific D_2 agonist quinpirole (0.5 mg/kg) produced a small but statistically significant decrease in hippocampal ACh release [$F(1,10)=10.51$, $p=0.009$]; examination of Fig. 17 suggests, however, that this may be a spurious statistical finding. Although CY 208-243 increased interstitial concentrations of ACh in the hippocampus, it did not appreciably activate the rats' locomotion; in contrast, the D_2 agonist had no obvious effect on ACh release while it elicited behavioural stereotypies that lasted approximately 1.5 h (data not shown).

The indirect DA agonist AMPH (2.0 mg/kg) also increased hippocampal ACh release compared to vehicle injections [$F(1,7)=42.44$, $p<0.001$], to a maximum of 250%

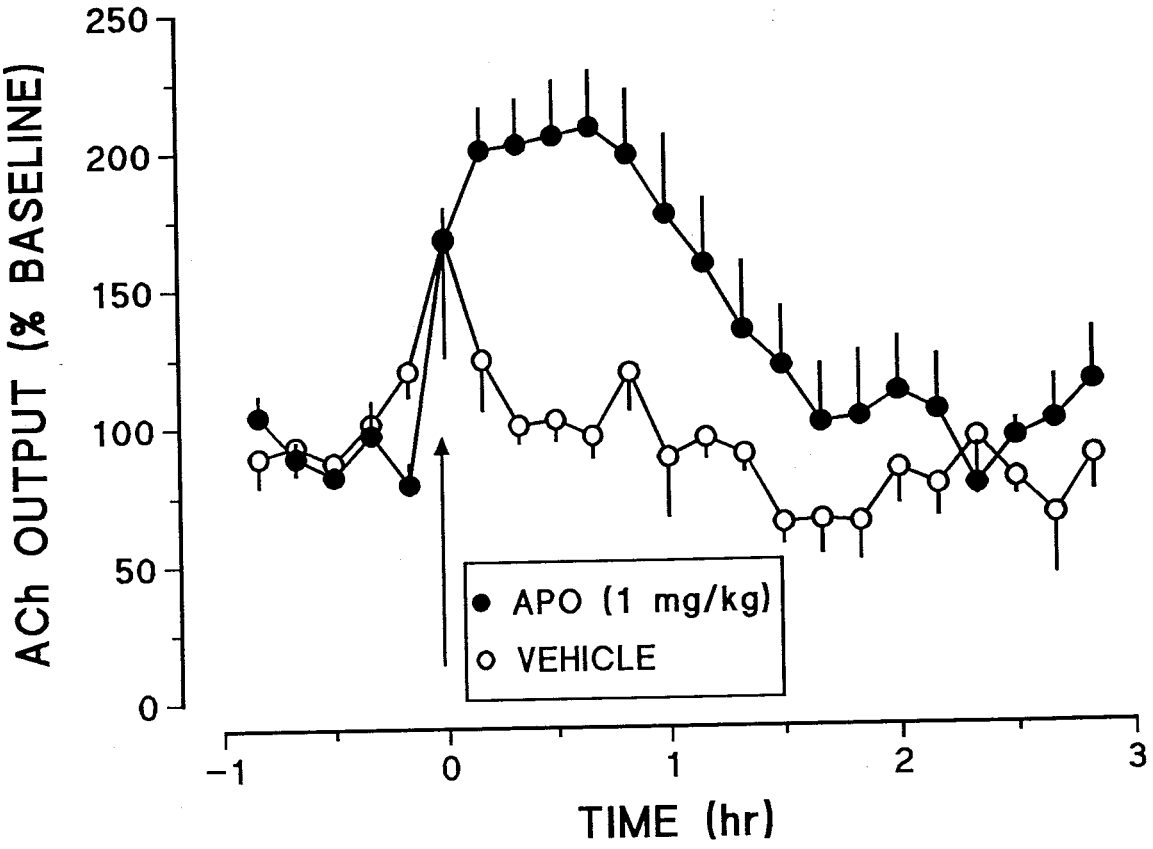


Figure 16. Hippocampal dialysate values of ACh after injection of apomorphine (APO; 1.0 mg/kg; closed circles; n=6) or vehicle (open circles; n=4). Data points represent group means ± S.E.M.

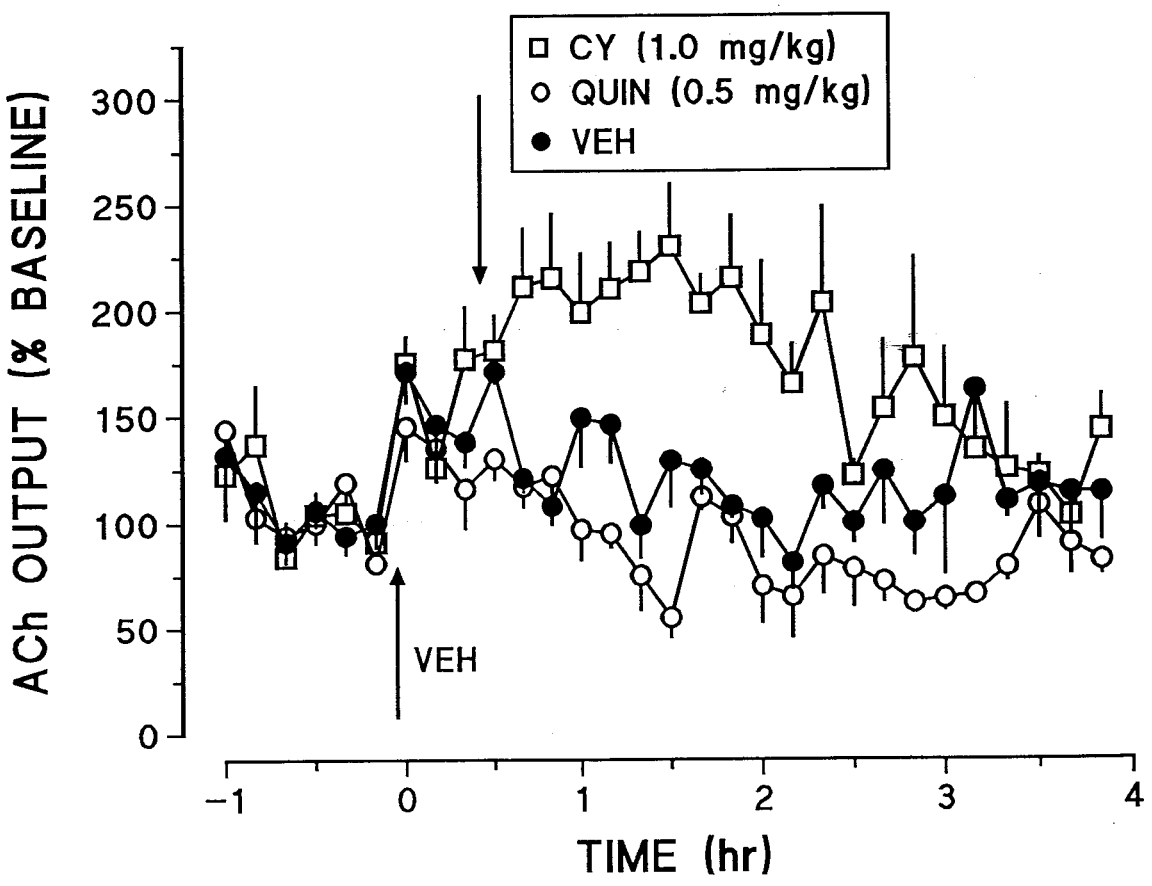


Figure 17. Hippocampal dialysate values of ACh after injections of CY 208-243 (CY; 1.0 mg/kg; open squares; n=5), quinpirole (QUIN; 0.5 mg/kg; open circles; n=7) or vehicle (closed circles; n=5). All groups were injected with vehicle 30 min prior to the second injection. Data points represent group means ± S.E.M.

baseline for about 3 h (Fig. 18). The D_1 receptor antagonist SCH 23390 (0.3 mg/kg), injected 30 min prior to AMPH, significantly attenuated AMPH's effect on hippocampal ACh release (170% *vs.* 250% maximal values; [$F(1,8)=22.06$, $p=0.002$; Fig 18A]). The AMPH-induced response following SCH 23390 pretreatment was not significantly different from that after either the control vehicle treatment [$F(1,9)<0.001$] or the SCH 23390/vehicle treatment [$F(1,8)=1.79$]. Compared to the control vehicle treatment (see Fig. 17), SCH 23390 significantly decreased hippocampal ACh release [$F(1,7)=8.29$, $p=0.024$]. The D_1 antagonist also blocked AMPH-induced behavioural hyperactivity (data not shown).

In contrast, the D_2 antagonist raclopride did not significantly attenuate the effect of AMPH on hippocampal ACh release (Fig. 18B). AMPH significantly increased ACh release after raclopride pretreatment, compared to both raclopride alone [$F(1,10)=16.60$, $p=0.002$] and to the vehicle control [$F(1,10)=6.21$, $p=0.032$]. The AMPH-induced increase in ACh release was not significantly decreased by raclopride pretreatment [$F(1,9)=2.02$]; it should be noted, however, that a significant treatment x time interaction was found [$F(9.89,88.99)=3.84$, $p<0.001$], possibly due to the larger blockade in the first hour than than that later in the time course (Fig. 18B). Raclopride also significantly decreased basal release of ACh, compared to the control vehicle injections [$F(1,8)=13.54$, $p=0.006$]. The D_2 antagonist completely blocked AMPH-induced behavioural hyperactivity in four of the seven rats, while three rats showed slight stimulation beginning in the second hour after AMPH injection (data not shown).

As shown in Fig. 19, AMPH (10 uM) applied locally through the dialysis membrane for 1 h did not significantly affect ACh release in the hippocampus [$F(2.89,8.68)=0.86$].

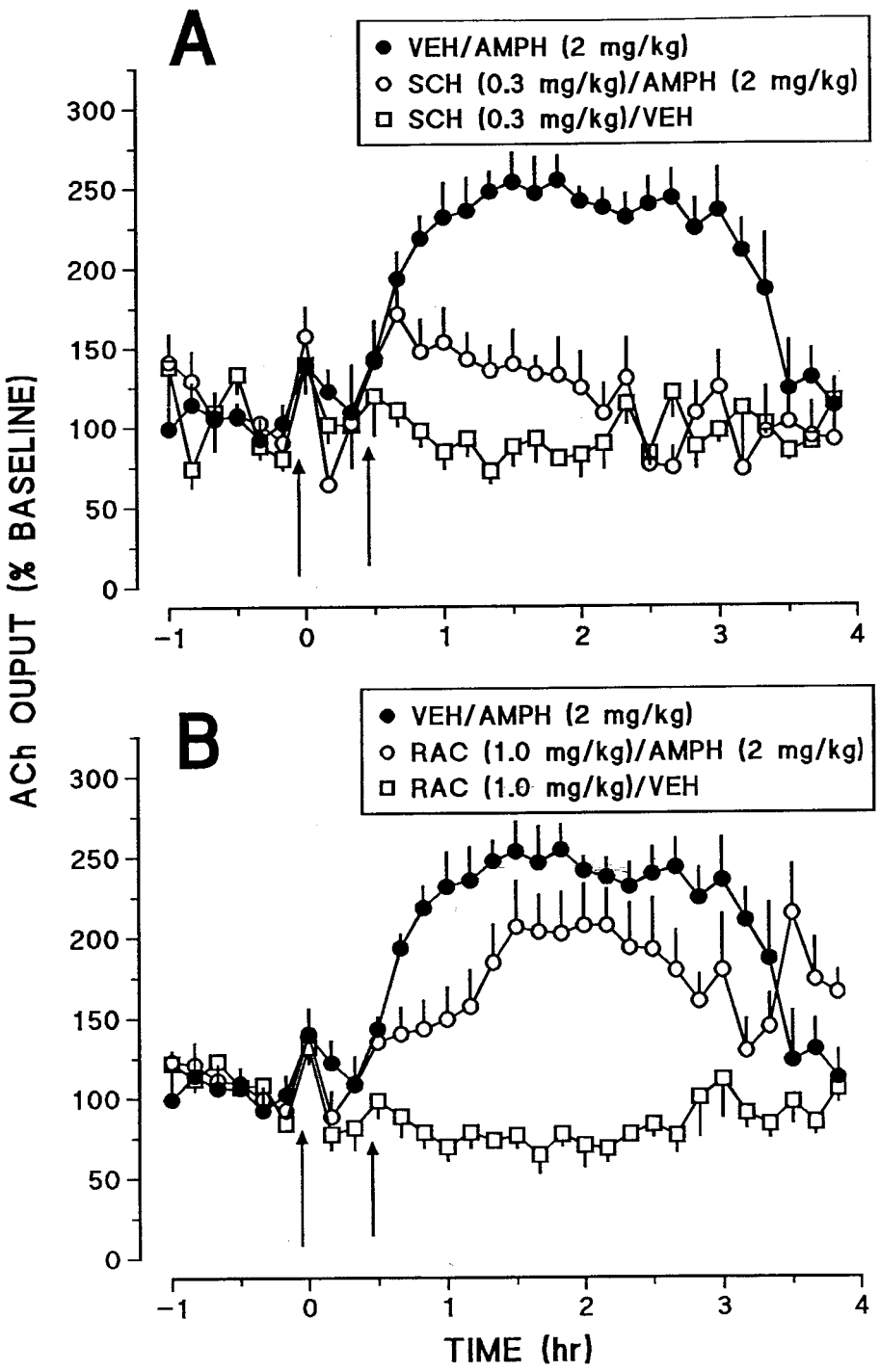


Figure 18. Hippocampal dialysate values of ACh after injections of *d*-amphetamine (AMPH; 2.0 mg/kg), SCH 23390 (SCH; 0.3 mg/kg; Fig. 3A), raclopride (RAC; 1.0 mg/kg; Fig. 3B) or vehicle in the following combinations: AMPH 30 min after vehicle injection (closed circles in A and B; n=4), AMPH 30 min after SCH 23390 (open circles in A; n=6), AMPH 30 min after raclopride (open circles in B; n=7), vehicle 30 min after SCH 23390 (open squares in A; n=4), and vehicle 30 min after raclopride (open squares in B; n=5). Data points represent group means ± S.E.M.

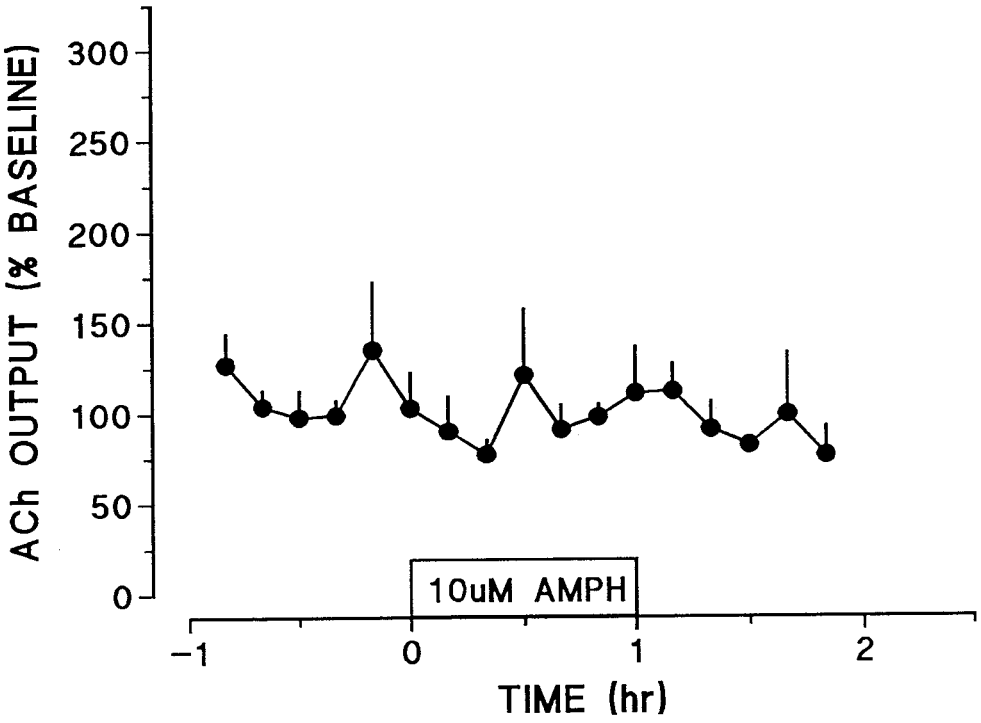


Figure 19. Effect of 10 *u*M *d*-amphetamine (AMPH) applied through the dialysis membrane for 1 h on hippocampal ACh output (n=4). Data points represent group means ± S.E.M.

(D) DISCUSSION

The results presented here suggest that the basal forebrain cholinergic projection to the hippocampus is regulated by DA acting primarily at D_1-type receptors. This conclusion is based on the findings that the indirect DA agonist AMPH, the DA receptor agonist apomorphine, and the D_1 receptor agonist CY 208-243 increased ACh release in the hippocampus, whereas the D_2 agonist quinpirole had a minor inhibitory effect. Furthermore, the D_1 antagonist SCH 23390 attenuated AMPH-induced increases of ACh release more effectively than did the D_2 antagonist raclopride. Nevertheless, a minor role of D_2 receptors in regulating hippocampal ACh release cannot be ruled out in view of the following findings: the D_2 antagonist reduced basal release of ACh (as did the D_1 antagonist), and it attenuated the effect of AMPH on hippocampal ACh release particularly during the first hour.

Regulation of hippocampally-projecting cholinergic neurons by DA

The conclusion that DA stimulates septohippocampal cholinergic neurons is consistent with recent reports of AMPH- and apomorphine-induced increases of hippocampal ACh release (Nilsson *et al.*, 1992; Imperato *et al.*, 1993b; 1993c). However, in contrast to the effect of AMPH on hippocampal ACh release observed here, Imperato *et al.* (1993c) described a very different time course of action of AMPH at the same dose, using experimental methods closely approximating those reported here. These authors reported AMPH-induced increases of hippocampal ACh release which return rapidly to baseline within 2 h. Although different routes of AMPH administration and a different strain of rats were used in the two studies (i.p. and Sprague Dawley, respectively, by Imperato *et al. vs.* s.c. and Wistar in the present experiments), this does not appear to explain the difference in time course, given that other authors using i.p. administration and Sprague Dawley rats (Nilsson *et al.*, 1992) report a time course

similar to that demonstrated here. The source of this difference in time course of AMPH's effects on hippocampal ACh release remains to be determined.

Although the data presented here are consistent with an important role of DA in regulating hippocampal ACh release, a role of other monoamines cannot be ruled out. As indicated earlier, AMPH releases noradrenaline and serotonin as well as DA (Kuczenski and Segal, 1989) and these other transmitters may therefore contribute to AMPH's effects on hippocampal ACh release, and account for the incomplete blockade of this effect by DA receptor antagonists. In support of this, it has been suggested that serotonin may increase release of ACh in the hippocampus (Bertorelli *et al.*, 1992; Maura and Raiteri, 1986; Ohue *et al.*, 1992); less information is available regarding noradrenergic regulation of hippocampal ACh.

In contrast to the data reported here, the results of earlier investigations led to the conclusion that DA was inhibitory on septohippocampal cholinergic neurons. It has been reported, for example, that apomorphine and a DA analogue decrease ACh turnover in the hippocampus, while intraseptal injections of haloperidol or 6-OHDA lesions increase this measure (Costa *et al.*, 1983; Robinson *et al.*, 1978; 1979). It is becoming increasingly clear, however, that drug-induced changes in regional ACh release as estimated by *in vivo* microdialysis have no predictable relationship to the effects of the same compounds on ACh turnover in the same brain structures (Chapter III; Costa *et al.*, 1983; Nilsson *et al.*, 1992; Robinson *et al.*, 1978; 1979). This suggests that turnover rate is not necessarily coupled to release.

Anatomical considerations

Anatomical evidence provides support for dopaminergic interactions with hippocampally-projecting CBC neurons. The medial septal nucleus and the nucleus of the diagonal band of Broca contain DA receptors, with a higher density of the D_1 receptor subtype than the D_2 subtype (Zilles *et al.*, 1991). Catecholaminergic terminals

synapse on identified cholinergic perikarya in the medial septum/diagonal band (Milner, 1991), although the majority of these afferents appear to be noradrenergic rather than dopaminergic (Lindvall and Stenevi, 1978; Moore, 1978). In addition, dopaminergic afferents innervate the lateral septum (Lindvall and Stenevi, 1978; Moore, 1978) which communicates directly with the medial septum (Swanson and Cowan, 1979); thus, a multisynaptic pathway within the basal forebrain may mediate the effects of DA on hippocampally-projecting cholinergic neurons.

DA may also have a transmitter role within the hippocampus (Bischoff *et al.*, 1979). In support of this hypothesis, dopaminergic innervation (Scatton *et al.*, 1980; Verney *et al.*, 1985) along with D_1 and D_2 receptors (Bouthenet *et al.*, 1987; Dewar and Reader, 1989; Mansour *et al.*, 1991) have been identified in the hippocampus. However, it is unlikely that the actions of DA on septohippocampal cholinergic neurons are mediated within the hippocampus, because of the demonstrated lack of effect of hippocampally-applied AMPH on ACh release. It is unlikely that this negative finding was due to insufficient dosage: AMPH is known to be permeable through the dialysis membrane used here (Nomikos *et al.*, 1990), and the concentration of AMPH chosen for this experiment has potent effects on dialysate concentrations of both DA and ACh in the striatum, as shown previously using identical methodology (Westerink *et al.*, 1990). Although it appears from the present experiment that DA released by AMPH is not acting within the hippocampus to increase ACh release, it has been reported previously that millimolar concentrations of locally applied DA increased hippocampal ACh release (Ohue *et al.*, 1992). The results of the latter experiment are suspect, however, given the high concentrations of DA applied, and the inappropriate perfusate and dialysis conditions (de Boer *et al.*, 1990a; Benveniste and Hüttemeier, 1990).

DA receptor subtype(s) mediating the dopaminergic regulation of septo-hippocampal cholinergic neurons

In general, the effects of specific D_1 and D_2 agonists and antagonists on hippocampal ACh release are very similar to those observed in the cortex (Chapter III). One minor difference between the microdialysis results obtained in the hippocampus and cortex is the modest inhibition of hippocampal ACh release observed here, compared to the lack of effect of D_2 receptor agonists on cortical ACh release. Experiments comparing dose-response effects of D_2 agonists on cortical and hippocampal ACh release are needed to determine if this discrepancy is simply due to differential sensitivity of these cholinergic projections to D_2 agonists, or whether it represents a qualitative difference in the dopaminergic regulation of these systems. Although it is possible that such differences exist, D_1 receptors are clearly more important than D_2 receptors in regulating ACh release in these brain regions.

The importance of the D_1 receptor subtype in mediating the effect of DA on hippocampal ACh release is supported by several other recent reports (Imperato *et al.*, 1993b; 1993c). However, these authors also reported a significant excitatory effect of the D_2 agonist quinpirole (Imperato *et al.*, 1993a). Quinpirole (0.5 mg/kg) was found to increase ACh release in the hippocampus to approximately 180% of basal release, whereas in the experiments reported here this same dose was found relatively ineffective, causing a small decrease of hippocampal ACh release. Given that a major strength of *in vivo* microdialysis is the ability to monitor the time course of action of pharmacological treatments, it is unfortunate that Imperato and colleagues (Imperato *et al.*, 1993b; 1993c) presented data from only one time point, which they described as the peak effect. Without a comparison of the full time course of action of the D_2 agonist, the basis of the difference between these previous data and those reported here cannot be determined. Given the variability of hippocampal ACh release over time (see Chapter II and the response of vehicle injected rats in Fig. 17), it is not only desirable,

but perhaps indeed necessary, to compare the effect of a drug over time with reference to a vehicle injection.

In conclusion, the data reported here suggest that hippocampally-projecting CBC neurons, similar to those that are cortically-projecting, are stimulated by DA acting primarily at D_1-type receptors that are located outside the hippocampus. This is consistent with the hypothesis that the CBC, which innervates both the hippocampus and cortex, may act as a functional unit at least with respect to its regulation by DA.

VI. SELECTIVE ENHANCEMENT OF CORTICAL, BUT NOT HIPPOCAMPAL, ACETYLCHOLINE RELEASE DURING THE ANTICIPATION AND CONSUMPTION OF A PALATABLE MEAL

(A) INTRODUCTION

Previous authors have proposed that CBC neurons may play important roles in learning and memory (Collerton, 1986; Hagan and Morris, 1988), attention (Muir *et al.*, 1994; Pang *et al.*, 1993; Voytko *et al.*, 1994), or arousal as defined electroencephalographically (Semba, 1991; Smythe *et al.*, 1992) or behaviourally (Chapter II; Collier and Mitchell, 1967). ACh released from these projections may influence cognition through facilitation of neuronal responsiveness to afferent signals, including sensory stimuli (Hars *et al.*, 1993; Metherate and Ashe, 1993; Tremblay *et al.*, 1990).

ACh has also been implicated in the regulation of conditioning-related neuronal responses. For example, frontal cortex neurons in the rat exhibit a discriminative response to conditioned stimuli; cortically-applied muscarininc receptor antagonists or lesions of CBC neurons attenuate these responses (Pirch *et al.*, 1992; Rigdon and Pirch, 1986). Discriminative responses have also been measured in unidentified neurons in the basal forebrain (Pirch, 1993). The conditioned stimulus (CS+) was a tone followed by rewarding medial forebrain bundle stimulation in this paradigm, although food following the CS+ could also be used to condition the discrimination in the cortex (as noted in Rigdon and Pirch, 1986).

More complex behavioural studies in monkeys have also suggested that responses of neurons in the basal forebrain are "context-dependent" (Richardson and DeLong, 1990) or "reinforcement-related" (Wilson and Rolls, 1990). For example, the increased firing of nucleus basalis neurons during the choice phase of a rewarded task was not found to be specific for the motor or sensory aspects of the task (Richardson and DeLong, 1990).

These researchers thus concluded that the responses of basal forebrain neurons may reflect transient increases in arousal or decision-making processes. Wilson and Rolls (1990) used a different task and described basal forebrain neurons which responded to sensory stimuli that, through learning of different contingencies, had come to signal the availability of reinforcement (access to fruit juice). Together, these data suggest that neurons in the basal forebrain may encode the learned reinforcement value, or significance, of stimuli. Although this theory is consistent with the proposed roles of forebrain ACh, the experiments described above did not identify the basal forebrain neurons under examination as being cholinergic, or as to their projection sites.

To assess the possibility that cortically- and/or hippocampally-projecting cholinergic neurons may be involved in signalling the learned reinforcement value of stimuli, *in vivo* brain microdialysis was used in the present experiments to measure ACh release in the frontal cortex and hippocampus of unrestrained, non-food-deprived rats trained to drink a palatable liquid chocolate meal. If CBC neurons are involved in learning about the availability of a reward, ACh release in the frontal cortex and/or hippocampus of trained rats might be expected to be augmented during anticipation and consumption of the reward, compared to naive or non-rewarded animals.

(B) MATERIALS AND METHODS

Experimental subjects

Experiments were performed on 40 male Long-Evans rats (Charles Rivers, Quebec). Rats which were subjected to behavioural training (n=27) weighed 275 $\pm$ 33g at the start of training, and 336 $\pm$ 30g at the time of surgery. Those run as naive controls (n=13) weighed 315 $\pm$ 12g at the time of surgery. The rats were housed individually under a 12:12 h light-dark cycle, and unless otherwise stated, had free access to food and water.

Behavioural training

Rats were trained in a Plexiglas chamber (40x30x40 cm) to drink a chocolate-flavoured liquid meal (Sustacal; Mead Johnson). The chamber was divided into two compartments (24x30x40 cm and 16x30x40 cm) by a removable plastic mesh screen. At one end of the chamber, in the smaller compartment, Sustacal was available through a 30 ml drinking tube.

On initial exposure to the chamber, with the screen absent, each rat was given 1 h to explore the chamber and to taste the chocolate solution. The rat was then returned home and water-deprived overnight. On all subsequent training sessions, the rat was placed in the chamber for 20 min (the "anticipatory" period), separated from the Sustacal by the mesh screen. The screen was then removed, and the rat was allowed 20 min in which to drink the Sustacal (consummatory period) and explore the entire chamber. At the end of the session, the rat was returned to its home cage. With the exception of the overnight water-deprivation following the first training session, rats had free access to food and water in their home cages throughout the period of the experiment. Rats were thus trained over a period of 14 days. Training took place once a day, between 1100h and 1400h. *In vivo* microdialysis was performed on day 14 of training.

Two groups of rats were run concurrently as controls. A group of "non-rewarded" rats was trained as described above, the exception being that throughout training these animals had access to water when the screen was removed. Another group (naive) consisted of animals that were first introduced into the experimental chamber, where Sustacal was available, on the day of dialysis.

Surgery and microdialysis

Brain microdialysis was performed essentially as described in Chapters III and V. On the afternoon of the 12th day of training, the rat was anaesthetized with Equithesin, and a transverse microdialysis probe was implanted stereotaxically into the frontal cortex

or dorsal hippocampus. Dialysis probes were made of acrylonitrile fibre (Chapters IV and V) and had active surface lengths of 6.8mm (hippocampus) and 10.9mm (cortex). Following surgery, rats were housed individually in Plexiglas cages with free access to food and water; they were allowed to recover and trained as previously. Dialysis sampling was initiated two days after surgery (on the 14th day of training) while the animal was in its home cage, approximately 3 h prior to the test session. The probe was perfused for 30 min before collection of samples to allow perfusate concentrations of ACh and Ch to equilibrate with those in the brain. After six baseline samples were collected in the homecage (baseline was defined behaviourally, with the animal resting, sleeping, or displaying low levels of quiet, non-exploratory behaviour such as grooming or chewing food), the rat was placed in the experimental chamber, and training carried out as described above. Dialysate samples were collected throughout this period, and for 150 min after the rat was returned to its home cage. Rats in the naive group were treated as described above on the day of the experiment, which was two days after probe implantation, and which represented their first exposure to the experimental chamber.

Assay of ACh

ACh was assayed by HPLC-ECD as described in Chapter IV.

Behavioural measures

Latency to drink Sustacal and volume consumed were recorded throughout training, and on the day of dialysis.

Statistical analyses

Volumes of liquid consumed and latencies to consumption on the day of dialysis were compared using univariate ANOVAs. Differences between trained rewarded rats

and the control groups were measured using Student's t-test, with Bonferroni correction for multiple comparisons.

Biochemical data were expressed as a percentage of baseline concentrations, 100% baseline being defined as an average of the final six values before rats were introduced into the experimental chamber. ANOVAs were used to test for group differences, time effects, and group x time interactions. In the latter two cases, Huynh-Feldt corrections were made to account for the use of time as a repeated measure. Differences in ACh release were assessed at individual time-points using univariate ANOVA. At each time-point, ACh concentrations in trained rats which received Sustacal were compared with those in both the trained non-rewarded rats and the naive rats.

(C) RESULTS

Behavioural responses

Other than ingestion of liquid, rewarded and non-rewarded rats displayed qualitatively similar behaviours. Most animals explored the chamber on the first days of training, but froze for several minutes when the screen was removed. On the day of dialysis, prior to removal of the screen, the rats displayed active exploratory behaviour such as running, burrowing in the chamber, sniffing, rearing, and nose-poking at the screen. On removal of the screen, rats in the trained rewarded and trained non-rewarded groups explored the previously closed area of the chamber. During the final minutes of this "consummatory period," many of the rats engaged in bed-making behaviour and began to sleep. Neither the latency to drink nor the amount of Sustacal consumed on the day of dialysis was different from the previous session, suggesting that cerebral perfusion did not adversely affect behaviour. Rats in the naive group displayed behaviours similar to rats in the other groups during the first days of training, and

typically froze when the screen was removed, and were slow to enter the previously restricted area of the cage.

Latency to drinking and volume consumed

Latencies to drink and volumes consumed for trained rewarded rats from day three to day 14 are presented in Fig. 20. Behavioural data from rats with cortical or hippocampal probes were not found to differ significantly (two-way ANOVA) and therefore these data were pooled. During the first seven days of training, the volume of Sustacal consumed increased gradually, and thereafter remained at an average of approximately 6ml throughout the training. The latency to drinking decreased steadily during training. The volume of Sustacal consumed was reduced slightly on days 13 and 14, while the latency on day 13 increased slightly, possibly due to trauma associated with the dialysis surgery. All trained rewarded rats drank Sustacal during the training sessions post-surgery.

Average latencies to drink and volumes consumed on the test (dialysis) session are presented in Table 5. Trained rats which received Sustacal drank a significantly greater volume of liquid than either the non-rewarded or the naive controls. The latency to drink was significantly greater for the naive rats than for the trained, rewarded rats, but was not significantly different between trained rewarded and non-rewarded groups.

Rats in the trained non-rewarded group frequently tasted the water in the drinking tube, but drank little. The average volume consumed on days three to 12 was less than 0.5ml; however, the volume of water consumed increased to an average (of the rats which drank) of 2ml and 1.5ml on days 13 and 14, respectively. Of the six rats which drank on the day of dialysis, the latency ranged from 4-97s.

Seven out of 13 naive rats placed in the experimental chamber for the first time during the dialysis test session consumed some Sustacal (average volume, of the rats

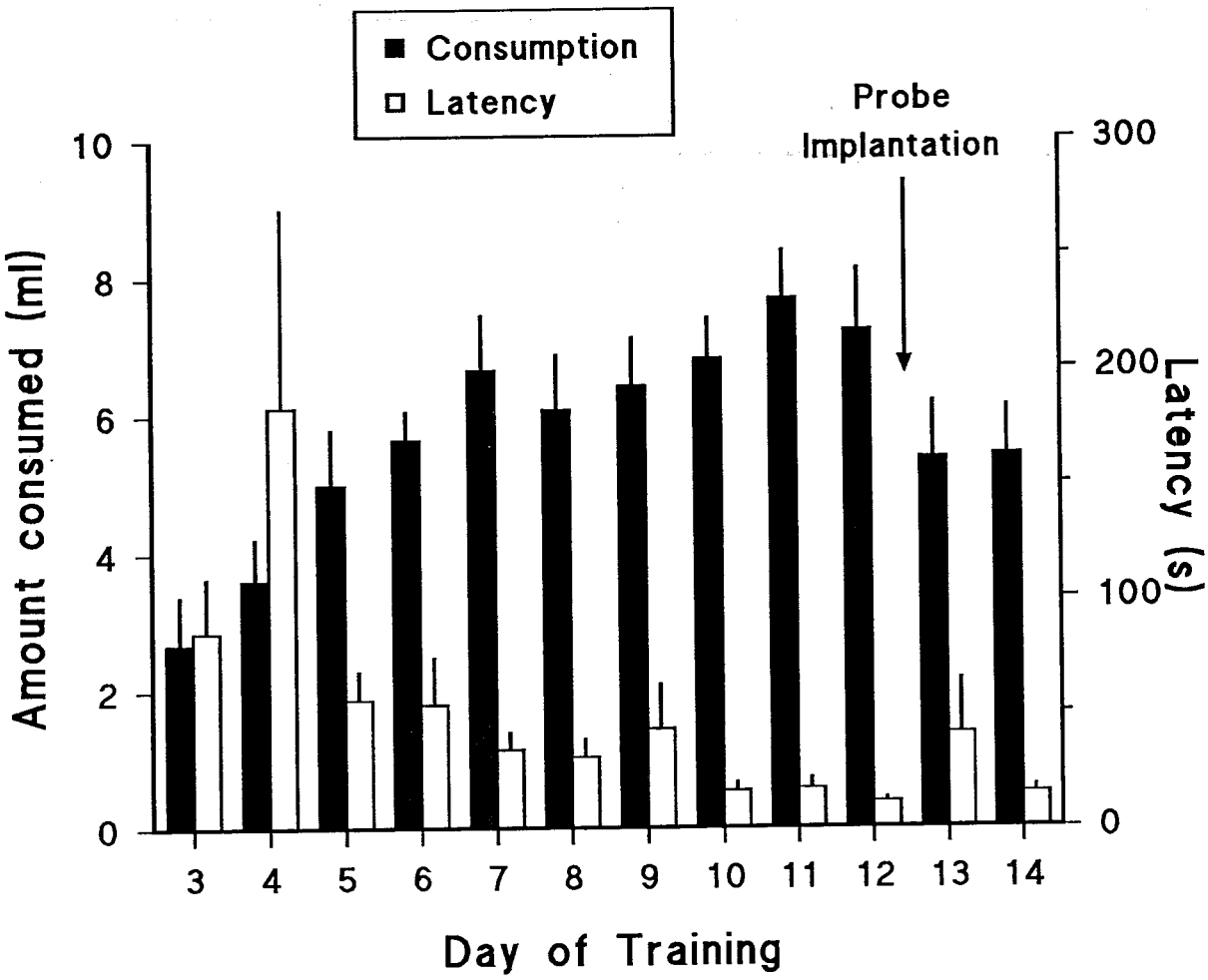


Figure 20. Volume of Sustacal consumed and latency to drink by trained rewarded rats during training days three to 14. Data are presented as mean ± S.E.M. (n=13).

Table 5. Consumption and latency to drinking during dialysis

Group	Volume (ml)	Latency (s)
Sustacal, trained	$5.4\pm0.7^{*,\#}$ (n=13)	$15\pm3^{*}$ (n=13)
Water, trained	0.8 ± 0.3 (n=14)	38 ± 14 (n=6)
Sustacal, naive	1.2 ± 0.4 (n=13)	395 ± 77 (n=7)

Data for volume consumed (average of all rats) and latency to drink (of rats which drank) on the day of dialysis is expressed as mean $\pm$ S.E.M. Univariate ANOVA revealed significant differences between group volumes [$F(2,37)=25.1$, $p<0.001$] and latencies [$F(1,23)=35.6$, $p<0.001$].

*Values are significantly different from the non-rewarded (water, trained) group. $p<0.01$.
$^{\#}$Value is significantly different from the naive group. $p<0.01$.

which drank, 2ml). Of these animals, two had hippocampal probes, and five had cortical probes. The latencies varied from 170-660s.

ACh release

The average basal output of ACh (mean ± S.E.M.) of all the animals used in these experiments was 61.65 ± 7.4 fmol/min in the frontal cortex (n=21) and 37.70 ± 3.9 fmol/min in the hippocampus (n=19). There was no significant difference in basal ACh output between groups with hippocampal probes [$F(2,16)=0.92$, $p=0.42$] or between groups with cortical probes [$F(2,18)=0.58$, $p=0.57$]. Because the basal outputs between groups were very similar, delta fmol/min calculations yielded very similar figures and identical statistical results to the percentage baseline calculations (data not shown).

Mean values of ACh release for each group of animals during the anticipatory and consummatory periods are presented in Table 6. It is evident from these averages, as well as from Figs. 21 and 22, that introduction of the rats to the experimental chamber differentially affected ACh release in the frontal cortex and hippocampus. Hippocampal ACh release (Fig. 21) increased to approximately 220% of baseline concentrations in all experimental groups, upon initial exposure to the chamber. No further increase occurred during the consummatory period in any group; ACh release decreased gradually over time and returned to baseline approximately 100 min after the rats were returned to their home cages. This effect of time was highly significant [$F(9.02,144.26)=24.16$, $p<0.001$]. However, ANOVA revealed no significant group difference [$F(2,16)=0.55$, $p=0.587$] or group x time interaction [$F(18.03,144.26)=1.11$, $p=0.352$] in the hippocampus.

In contrast, ACh release in the frontal cortex (Fig. 22) varied between the three groups of rats. On initial exposure to the experimental chamber, ACh release increased to approximately 300% of baseline in rats trained with Sustacal. ACh release continued to increase and was maximal during the first sample after removal of the screen, when Sustacal consumption was greatest. ACh release in both the trained non-rewarded group

Table 6. Average acetylcholine release during anticipatory and consummatory periods

Group	Acetylcholine release (mean % baseline ± S.E.M.) Anticipatory Period	Consummatory Period
HIPPOCAMPUS		
Sustacal, trained (n=6)	211±28	190±28
Water, trained (n=7)	204±8	181±10
Sustacal, naive (n=6)	231±10	201±19
CORTEX		
Sustacal, trained (n=7)	314±46	312±49
Water, trained (n=7)	224±30	187±33
Sustacal, naive (n=7)	184±7	169±15

ACh release was averaged over the two samples in each period.

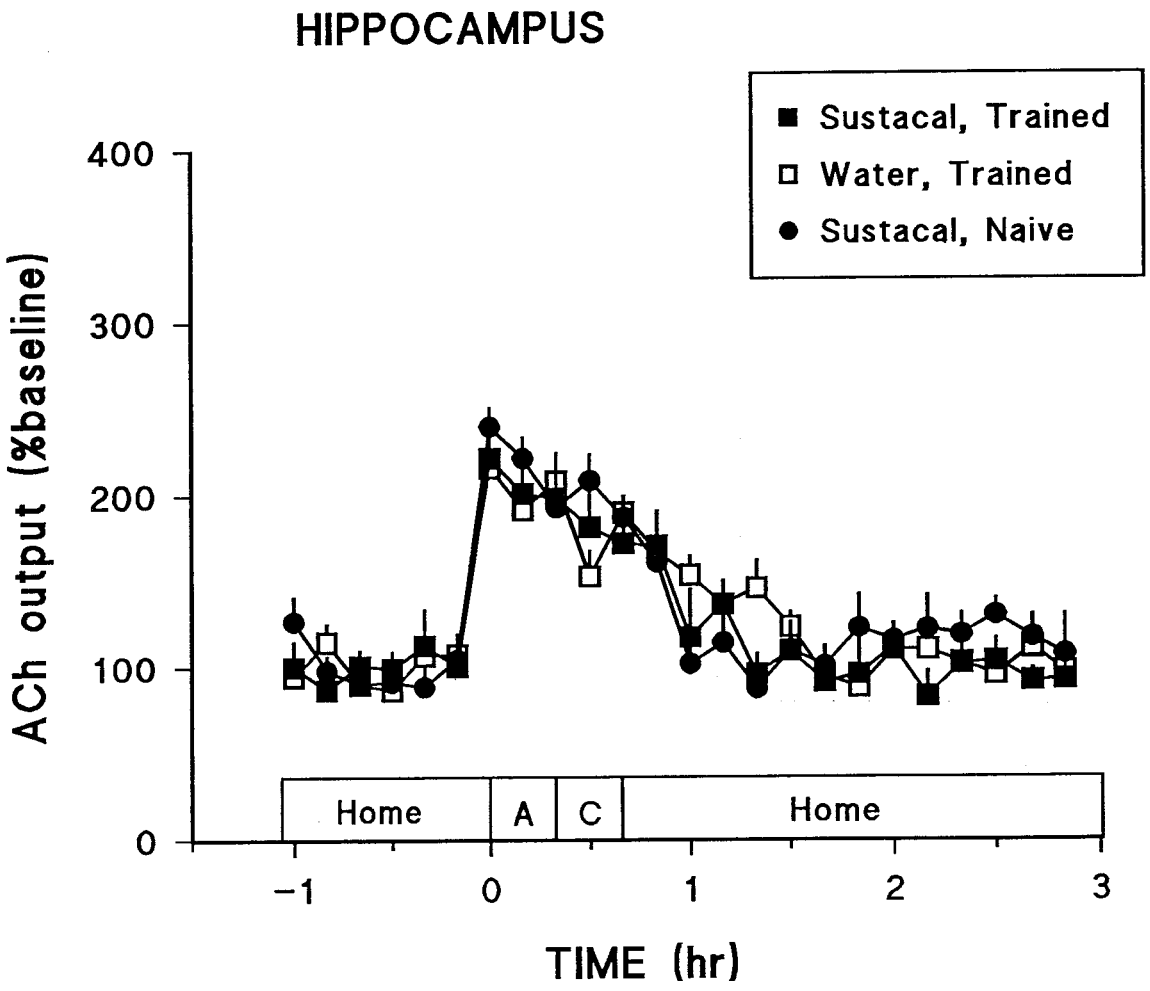


Figure 21. Dialysate concentrations of ACh in the hippocampus of trained rewarded rats (Sustacal; closed squares; n=6), trained non-rewarded rats (water; open squares; n=;7) and naive rats (closed circles; n=6) during the anticipatory (A) and consummatory (C) periods of the task. Data points represent group means ± S.E.M.

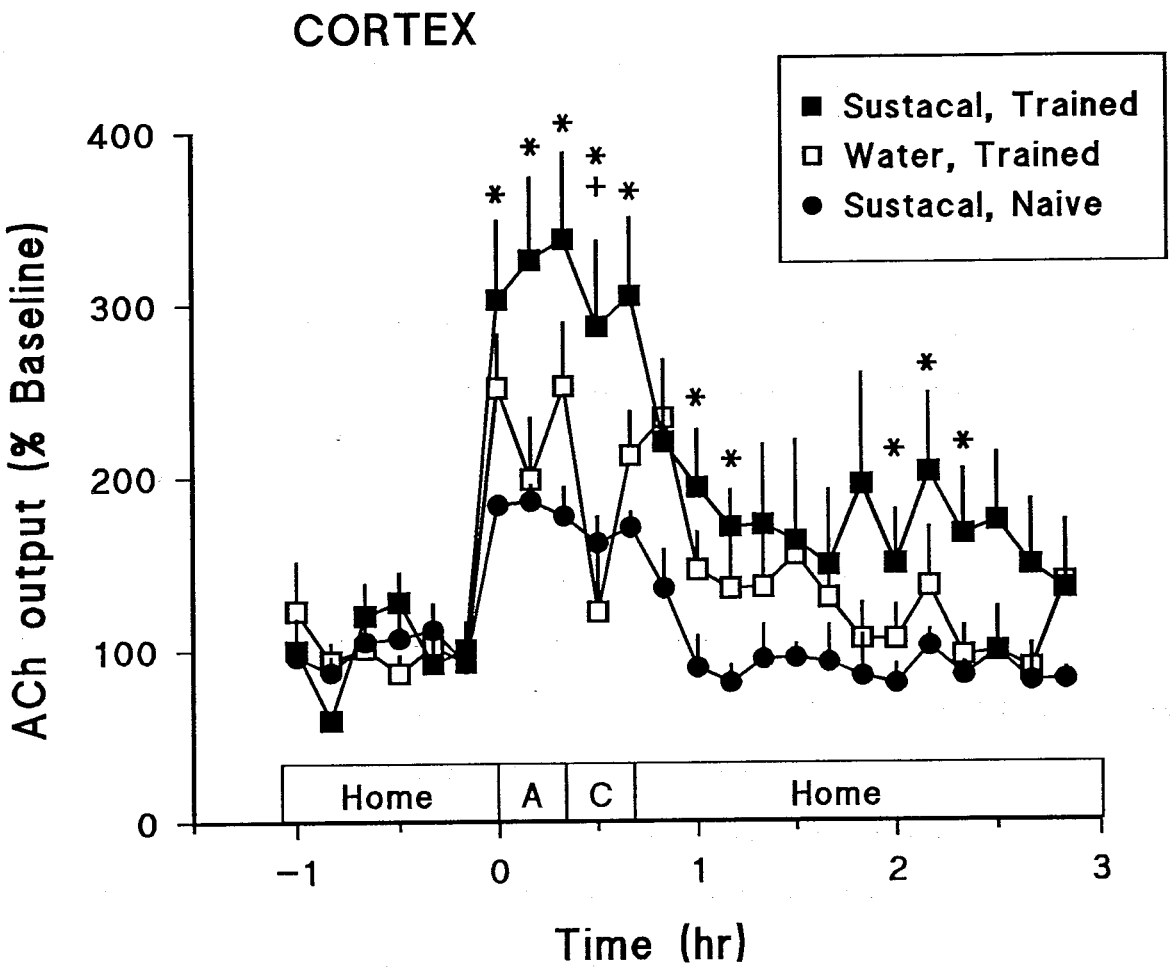


Figure 22. Dialysate concentrations of ACh in the cortex of trained rewarded rats (Sustacal; closed squares; n=7), trained non-rewarded rats (water; open squares; n=7) and naive rats (closed circles; n=7) during the anticipatory (A) and consummatory (C) periods of the task. Data points represent group means ± S.E.M. * significantly different from naive rats; + significantly different from trained non-rewarded (water) rats; $p<0.05$.

and the naive group also increased when the rats were in the experimental chamber, but these increases were smaller than that seen in the trained rewarded group. Two-way ANOVA revealed a significant difference in cortical ACh release between groups [$F(2,18)=4.27$, $p=0.030$] but no group x time interaction [$F(24.17,217.56)=1.21$, $p=0.237$]. The effect of time was again highly significant [$F(12.09,217.56)=14.35$, $p<0.001$]. Univariate ANOVA demonstrated differences between groups at several time-points, as noted in Fig. 22.

(D) DISCUSSION

The present results demonstrate that different patterns of ACh release occur in the frontal cortex and hippocampus of rats during the anticipation and consumption of a highly palatable meal. While ACh release in the hippocampus increased similarly in each group, regardless of previous training or the presence of a reward, ACh release in the frontal cortex occurred in a differential manner. That is, in the frontal cortex ACh release was greater in those rats trained to anticipate and consume a palatable reward.

The increased ACh release occuring during this task can be separated into two components. Both the hippocampus and cortex exhibit the first component, which is a training- and reward-independent increase. Only the cortex, however, exhibits the second component which is an increase over and above Component 1 and appears to be reinforcement-related. These results are notably similar to an earlier report concerning enhanced cholinergic transmission in the cortex of rabbits trained to press a lever for a water reward in response to auditory or visual cues, on different operant schedules of reinforcement (Rasmusson and Szerb, 1975; 1976). Cortical ACh release was increased in response to performing the behavioural task, in the five tested combinations of two cortical regions, two cues of differing sensory modality and two schedules of

reinforcement. However, a second, larger increase was also noted in the sensorimotor cortex of light-cued rabbits reinforced for low response rates. The two components of increased ACh release found in the present task are discussed below.

Component 1: Training- and reinforcement-independent increases of ACh release in cortex and hippocampus

The increased ACh release evident during this task in the hippocampus and in the cortex of naive and non-rewarded rats may be due to a number of variables. For example, forebrain cholinergic neurons are involved in arousal defined by electrophysiological (Semba, 1991; Smythe *et al.*, 1992) or behavioural (Chapter II; Collier and Mitchell, 1967) measures. Arousing stimuli, such as handling or injecting the animal, have also been shown to increase hippocampal and/or cortical ACh release (Chapter II; Dudar *et al.*, 1979; Nilsson *et al.*, 1990). The animals' behaviours in the present task suggested that they were aroused by being put into an environment different than their home cages (the experimental chamber), both when the situation was novel (naive group) and after they had repeated access to it (trained non-rewarded group). Thus, increases in arousal may explain the first component of the increased ACh release in the cortex and hippocampus.

Attention, defined operationally in behavioural studies, is also thought to involve forebrain cholinergic neurons. For example, excitotoxic lesions or pharmacological inhibition of the basal forebrain causes impairments in measures of attention (Olton *et al.*, 1988; Pang *et al.*, 1993; Robbins *et al.*, 1989a; Voytko *et al.*, 1994) which in some cases have been ameliorated by cholinergic agonists or tissue grafts (Muir *et al.*, 1992a; 1992b; 1994). In addition, cholinergic agonists improve measures of attention in Alzheimer's patients (Sahakian *et al.*, 1989; 1993) and the cholinergic receptor antagonist, scopolamine, disrupts attention in normal human subjects (Dunne and Hartley, 1985;

Wesnes and Warburton, 1983). It is possible, therefore, that increased attentiveness of the rats during the task may account for increased ACh release.

Given that factors such as arousal or attention may increase ACh release during behavioural tasks, independent of learning or memory *per se* (as in the naive groups), it is evident that attempts to study cholinergic correlates of learning and memory must carefully control for these factors. However, while arousal and/or attention may have contributed to the increased ACh release, these mechanisms cannot explain the significantly greater ACh release in the frontal cortex of those rats which were trained with Sustacal. This assertion is similar to that made by Rasmusson and Szerb (1975; 1976) in the interpretation of their results, which have been described above: although the general increase of cortical ACh release seen in all groups might be explained by some shared factor, such as the behavioural activity or "work expended" by the animal during the task, this factor could not account for the larger increase observed in the group which exhibited lower response rates.

Component 2: Reinforcement-related increase of ACh release in the cortex

The finding of an increase in cortical ACh release in the trained rewarded group, over and above the arousal/attention related increase, is consistent with reports of reinforcement-related neurons in regions of the basal forebrain which contain cortically-projecting cholinergic neurons (Richardson *et al.*, 1991; Wilson and Rolls, 1990). Studies have demonstrated neurons within the hippocampus and cortex which respond specifically during the delay between stimulus and reward in tasks which require remembering the position of a cue (Kojima and Goldman-Rakic, 1982; Watanabe and Niki, 1985); in contrast, few basal forebrain neurons are active during such delays (Richardson and DeLong, 1990). This suggests that while there is a role for the hippocampus and frontal cortex in remembering information during a delay, the cortical projection from the basal forebrain is involved specifically in recognition of those cues

which signal the reward. Thus, the context-specific alterations in cortical ACh release observed in the present experiment are consistent with a specific role of cortically-projecting CBC neurons in the recognition or anticipation of an impending food reward. In this regard, it is noteworthy that the group of rabbits exhibiting the largest increase in cortical ACh release in the experiments of Rasmusson and Szerb (1976) were those to receive significantly more reinforcements than any other group.

The present results demonstrate differences in task-induced ACh release in the cortex and hippocampus. It is possible that these differences could be explained on anatomical grounds. For example, combined anterograde tracing and immunohistochemical techniques have demonstrated that cholinergic neurons in the CBC and mesopontine tegmentum innervate the frontal cortex (Satoh and Fibiger, 1986). It is conceivable, therefore, that the group-dependent patterns of ACh release which occurred in the frontal cortex, but not in the hippocampus, were due in part to enhanced discharge of mesopontine neurons in response to the anticipation and presentation of the reward. However, the distribution of terminals in the frontal cortex arising from the laterodorsal tegmental nucleus is confined to the medial frontal cortex, at a level more ventral and posterior to the area of cortex dialysed in the present study. Further, there are no data to suggest that mesopontine cholinergic neurons are active specifically during the anticipation and presentation of a reward. It should also be noted, with regard to the anatomical specificity of "Component 2" of the ACh release in this task, that the results presented here for the frontal cortex are not necessarily representative of the entire neocortex. For example, Rasmusson and Szerb (1976) reported differences in the effects of a behavioural task on ACh release in the sensorimotor and visual cortices of rabbits.

Another possible explanation for the differences in cortical and hippocampal ACh release in this task is that the activities of cortically- and hippocampally-projecting cholinergic neurons can be differentially regulated. Previously, it has been shown that

pharmacological and physiological stimuli have nearly identical effects on ACh release in these structures (Chapters II, III and V) lending support to the hypothesis that the continuum of neurons that forms the CBC (Schwaber *et al.*, 1987) acts as a single functional unit. The present results show that this is not the case, and argue for a subdivision of this continuum on functional grounds.

The question then arises as to which input(s) to the basal forebrain differentially regulate cortically- and hippocampally-projecting CBC neurons in the present task. There is extensive evidence that dopaminergic mechanisms are involved in reward (Fibiger and Phillips, 1986). Dopaminergic mechanisms are also involved in the regulation of ACh release in the hippocampus and frontal cortex (Chapters III, IV, V). However, it seems unlikely that regulation of CBC neurons by DA can account for the differential ACh release in the cortex and hippocampus in this study, for the following reasons. In rats trained in the same paradigm as that used here, DA release increases in the nucleus accumbens; however, these increases occur during the consumption rather than during the anticipation of the reward (Fibiger, 1993). Given this difference, the increased ACh release observed in the present study is not likely the indirect result of increased DA release in the nucleus accumbens. In addition, it has been demonstrated that dopaminergic regulation of cortical and hippocampal ACh release is very similar (Chapters III and V).

Although the anatomical or transmitter-specific basis for the differential regulation of cortical and hippocampal ACh release in the present task is unknown, it is possible that the different patterns observed may have been due to the type of behavioural task employed. While there is evidence that both the hippocampus and frontal cortex are involved in learning (Hagan and Morris, 1988), the hippocampus has been implicated specifically in spatial memory (O'Keefe and Nadel, 1978). It is possible, therefore, that a task which depends specifically upon learning about the spatial orientation of a reward

might increase ACh release in the hippocampus, compared to a non-rewarded or naive control group.

VII. GENERAL DISCUSSION

The experiments presented in this thesis have demonstrated that ACh release in the frontal cortex and hippocampus varies depending on the animal's level of arousal. Cortically- and hippocampally-projecting neurons in the CBC are regulated in an excitatory manner by DA acting primarily at receptors of the D_1 subtype, probably located outside of the terminal regions of these neurons. Although cortical and hippocampal ACh release are affected very similarly by dopaminergic drugs, the anatomically continuous CBC can be functionally subdivided based on differential ACh release in the cortex and hippocampus of rats trained to anticipate and consume a palatable meal. The relevance of these findings with respect to both the technical aspects of cholinergic research, and to theories of forebrain cholinergic function, are discussed below.

(A) TECHNICAL CONSIDERATIONS OF CHOLINERGIC RESEARCH

Microdialysis represents an improvement over previously used *in vivo* sampling techniques, such as cortical cup or push-pull perfusion, by virtue of its ease of applicability to freely moving animals and its less traumatic tissue interaction. The sensitivity of the HPLC-ECD assay for ACh has also allowed the use of more physiological perfusion solutions (lower concentrations of cholinesterase inhibitor and calcium ions), while the on-line assembly makes data available with as little as a fifteen minute time delay. This latter advance is highly desirable for *in vivo* determination of ACh, given the labile nature of hippocampal and cortical ACh release and given that it is advantageous to know that a relatively stable baseline has been achieved before an experimental manipulation is initiated. The improvements noted above may account for

some discrepancies between the present results and those found previously using cortical cup or push-pull techniques. For example, Dudar *et al.* (1979) found that hippocampal but not cortical ACh release was responsive to sensory stimuli and Casamenti *et al.* (1987) reported D_2- but not D_1-mediated regulation of cortical ACh release.

In contrast, the factors accounting for discrepancies between the present results and those found previously using ACh turnover rate assays remain poorly understood. These methods are based on the proposition that steady-state kinetics can be applied to ACh turnover; however, the assumptions made underlying this proposition may be unfounded (Cheney and Costa, 1977) and may therefore limit the suitability of the ACh turnover measure as an indicator of cholinergic neuronal activity. The present microdialysis data and those of others, concerning the effects of dopaminergic drugs on hippocampal and cortical ACh release (Nilsson *et al.*, 1992), have no predictable relationship to the effects of the same compounds on ACh turnover in the same brain structures (Costa *et al.*, 1983; Robinson *et al.*, 1978; 1979). It is thus becoming apparent that turnover rate is not necessarily coupled to release and that the functional significance of ACh turnover is unknown.

To augment the substantial advantages over previous *in vivo* sampling techniques offered by dialysis, methodological refinements are continually being made to this technique. Statistical treatment and interpretation of the data is one area which requires improvement and standardization, especially in the case of forebrain ACh release which is highly labile and subject to large basal variations. Attempts to control for the individual variabilities in basal dialysate concentrations of a neurotransmitter have usually involved expressing these concentrations as percentages of baseline. This manipulation is probably appropriate if groups with substantially different average basal outputs are not compared. If such comparisons are to be made, it is more appropriate to use calculations such as the delta fmol/min (presented in Chapter IV) or absolute outputs using a basal value as a covariate of statistical comparisons (Moore *et al.*, 1993).

Although the alternative data description techniques noted above may allow more appropriate analyses of ACh microdialysis results, it remains necessary in any of these cases to define a "basal" concentration, or output, of neurotransmitter recovered from the brain region of interest in each animal. This is not a straightforward task in the case of ACh microdialysis given the correlation of ACh release and behavioural activity (Chapter II) and given the between-animal variability in behavioural activity over time. While "baseline" is usually defined as the average of three to six pre-treatment concentrations, the behavioural activity of the animals during this period is often not reported. Comparisons between research groups using ACh microdialysis would be facilitated if the definition of baseline were standardized, ideally including averaged values from an hour of sampling during sleeping and quiet waking behaviours, as in Chapter VI.

Future challenges to the improvement of the microdialysis technique for measurement of ACh are manifold. Most investigations into the principles of microdialysis have been carried out using catecholamine assays. The significance of the perfusion-related neurochemical "sink" (Benveniste, 1989; Blaha, 1991) remains to be characterized in the case of ACh; the possibility that a low concentration of the reversible cholinesterase inhibitor neostigmine in the perfusion solution might counteract this sink also requires examination. Further characterization of the correlation between ACh release and behavioural activity should also be a priority of basic ACh dialysis research, given that the ultimate research objectives to which ACh microdialysis can be applied include behavioural studies. An improved understanding of how ACh release is related to behaviour, how this relationship can be dissociated pharmacologically, and how it can be controlled for to allow unconfounded measurements of cognition-related cholinergic activity will probably depend on improved sensitivity and temporal resolution of the ACh assay coupled with more detailed behavioural characterizations.

(B) REGULATION OF BASAL FOREBRAIN CHOLINERGIC NEURONS

The results of Chapters II and VI are in agreement with the hypothesis that CBC neurons are involved in cognitive functions including arousal and attention. These functions may subserve the commonly accepted role of ACh in learning and memory (Hagan and Morris, 1988) and the proposed association of decreased cholinergic capacity with dementia (Collerton, 1986; Coyle *et al.*, 1983). The huge social and economic impact of dementing diseases such as Alzheimer's, in a society with a growing percentage of its population found in the most-affected age group, provides the mandate for investigating the regulation of CBC neurons.

Although the shortcomings of the cholinergic hypothesis of dementia are recognized (Fibiger, 1991), and despite the overall ineffectiveness of cholinergic therapies in treating dementia (Holttum and Gershon, 1992), the search continues for a pharmacological agent which stimulates forebrain cholinergic systems to ameliorate cognitive deficits. For example, it has been hypothesized that attempts to treat dementias with cholinergic receptor agonists or cholinesterase inhibitors may be unsuccessful because the normal spatial and temporal patterning of cholinergic transmission is not maintained by such treatments (Drachman *et al.*, 1982). In normal circumstances, ACh facilitates the activity of cortical neurons responding to afferent neural transmission, and an approximate temporal coincidence of the cholinergic stimulation with the afferent stimulus appears to be necessary for this increase in the "signal" (Hars *et al.*, 1993; Metherate *et al.*, 1987; Tremblay *et al.*, 1990). Conversely, using cholinergic agonists to improve cognitive deficits may increase the "noise" in the system by increasing cholinergic tone in a temporally non-selective manner. A similar situation may apply in the hippocampus, where ACh facilitates long-term potentiation (Blitzer *et al.*, 1990; Hunter *et al.*, 1994) and enhances excitatory postsynaptic potentials elicited by afferent stimulation (Markram and Segal, 1990).

Based on the above arguments, it has been suggested that a more promising pharmacological approach to the treatment of dementia would be to amplify the activity of remaining CBC neurons without disrupting the normal patterning of cholinergic transmission (Sarter *et al.*, 1990). These authors review evidence suggesting that pharmacologically modulating the inhibitory GABAergic regulation of CBC neurons might have such a therapeutic action. Could similar arguments also apply to the dopaminergic regulation of CBC neurons? Together with the cholinergic hypothesis of dementia, the results of Chapters III-V suggest that dopaminergic agonists, especially those acting at the D_1 DA receptor subtype, may enhance cognition *via* stimulation of forebrain cholinergic function.

The above hypothesis, that dopaminergic drugs may act as cognition enhancers, has not received unequivocal support from the results of previous research. Although AMPH improves the performance of both animals and humans in a variety of learning and memory tasks (Doty and Doty, 1966; McGaugh, 1973; Packard and White, 1989; Quartermain *et al.*, 1988; Soetens *et al.*, 1993; Strupp *et al.*, 1991; Weingartner *et al.*, 1982), AMPH has also been reported to have no effect or deleterious effects on the performance of other tasks (Beatty and Rush, 1983; Beatty *et al.*, 1984; Ennaceur, 1994; Kesner *et al.*, 1981). These apparently contradictory results may be reconciled in several ways. Kesner and colleagues have suggested that arousal, such as that induced by AMPH (Fairchild *et al.*, 1967), is detrimental for the induction of short-term memory while it facilitates the consolidation required for long-term memory (Kesner, 1973; Kesner *et al.*, 1981). It has also been proposed that AMPH-induced changes in sensorimotor functions or attention may actually account for what had been reported as AMPH-induced memory effects (Ennaceur, 1994; Beatty and Rush, 1983; Burešová and Bureš, 1982).

The results of more specific dopaminergic drugs on cognitive tasks are no less ambiguous. Cognitive deficits after withdrawal of L-dopa in Parkinson's patients have

been reported (Lange *et al.*, 1992), as have retention-improving effects of apomorphine in rats (Grecksch and Matthies, 1981). Conversely, Burešová and Bureš (1982) and Chrobak and Napier (1992) found no effect of apomorphine on rats' performance. More recently, researchers have begun to question which DA receptor subtype(s) may be involved in cognition; given the inconsistencies noted above, it is perhaps not surprising that conflicting evidence has been reported (Castellano *et al.*, 1991; Chrobak and Napier, 1992; Levin *et al.*, 1990). For example, although a D_2 but not a D_1 agonist improved the performance of rats in a radial maze (Packard and White, 1989), cortical injections of a D_1 but not a D_2 antagonist have recently been reported to cause cognitive impairments in monkeys (Sawaguchi and Goldman-Rakic, 1994).

The lack of concensus as to the role of DA in cognition, as noted above, may be due to variations among studies including the different dopaminergic drugs, cognitive tasks, experimental measures and interpretation of results. The ambiguities may also reflect a complex, regionally-specific involvement of DA in cognitive functions that has not been adequately assessed by the majority of behavioural studies thus far conducted using systemic administration of drugs.

Irrespective of whether or not systemically applied dopaminergic drugs might have clinical applications as cognition enhancers, the significance of the dopaminergic regulation of CBC neurons in the normally functioning nervous system is of great theoretical interest. As discussed previously, ACh in the cortex and hippocampus appears to act by accentuating the activity of afferent transmission with which its release is paired. Stimulation of cortical and hippocampal ACh release by DA might thereby be a means by which dopaminergic transmission could signal the saliency of afferent transmission to the hippocampus and cortex. Indeed, mesotelencephalic DA systems are known to be activated by stressful as well as rewarding stimuli (Abercrombie *et al.*, 1989; Bertolucci-D'Angio *et al.*, 1990; Deutch and Roth, 1990; Di Chiara and Imperato, 1988; Fibiger and Phillips, 1986). The increased DA release may be partially responsible

for the reported activation of basal forebrain neurons during the presentation of cues previously paired with aversive or rewarding stimuli (Richardson and DeLong, 1990; Whalen *et al.*, 1994; Wilson and Rolls, 1990). The extent to which the dopaminergic regulation of CBC neurons is involved with cholinergic facilitation of afferent transmission in the cortex and hippocampus remains to be determined. In addition, given the interactions of cholinergic transmission with noradrenaline and serotonin in learning and memory (Decker and McGaugh, 1991), the possible regulation of CBC neurons by these other diffusely-projecting, ascending monoaminergic systems merits future research using the microdialysis technique.

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