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# Antagonism by Abecarnil of Enhanced Acetylcholine Release in the Rat Brain During Anticipation But Not Consumption of Food

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GHIANI, C. A., DAZZI, E. MACIOCCO, G. FLORE, G. MAIRA AND G. BIGGIO. *Antagonism by abecarnil of enhanced acetylcholine release in the frontal cortex and hippocampus of rats during anticipation but not consumption of food.* PHARMACOL BIOCHEM BEHAV **59**(3) 657–662, 1998.—Changes in the extracellular concentration of acetylcholine (ACh) were evaluated in the prefrontal cortex and hippocampus of freely moving rats habituated for 35 days to consume their daily meal during a fixed 2-h period. During the 40 min immediately before presentation, ACh output increased by 49 and 55% in the prefrontal cortex and hippocampus, respectively. ACh release increased further during the first 40 min of consumption phase in the prefrontal cortex (+220%) and hippocampus (175%). Administration of abecarnil (0.1 mg/kg, *IP*) 40 min before food presentation prevented the increase in ACh output in both brain regions during the anticipatory phase. In contrast, although abecarnil reduced the ACh content achieved during the consummatory phase, it did not prevent the increase in ACh release in the prefrontal cortex or hippocampus induced by food intake. Finally, the binding of [$^{35}$S]TPBS to cerebral cortex, hippocampus, or septum of rats killed 20 min before food presentation was significantly higher than the values for animals killed 2 h after food presentation. These results suggest that during ingestive behavior ACh release is regulated by at least two independent mechanisms: one, associated with the anticipatory phase, that is sensitive to the activation of $GABA_A$ receptors, and a second, associated with the consummatory phase, that is insensitive to abecarnil. © 1998 Elsevier Science Inc.

Acetylcholine release  Hippocampus  Cerebral cortex  Feeding  Starvation  Abecarnil  Emotional state

PHARMACOLOGICAL evidence suggests that acetylcholine (ACh) is important in the modulation of various brain functions including learning and memory, attention and arousal, the control of locomotor activity, and the response to stressful stimuli (8,13–17,19,24,25). The release if ACh in various regions of rat brain also can be potentiated by ingestive behaviors. Thus, unlimited feeding in rats deprived of food for 20 h results in a marked increase in the extracellular concentration of ACh in the nucleus accumbens (23). Moreover, ACh release was increased in both the frontal cortex and hippocampus of rats trained to consume a palatable meal (20). Different patterns of ACh release in the frontal cortex were apparent during the anticipation and consumption of such food. Indeed, there appears to be a second component regulating ACh release in the frontal cortex of rats trained to receive a palatable reward, in addition to that responsible for the increase in ACh release related to the behavioral stimulation associated with the anticipatory phase.

To clarify further the relation between cholinergic function and emotional and behavioral processes associated with ingestive behavior, we have now investigated the time course of the changes in the basal extracellular concentration of ACh in the brains of freely moving rats trained for 35 days to eat their normal daily meal in a period of 2 h. We measured ACh output in the frontal cortex and hippocampus every 20 min for 2 h during both the anticipatory and consummatory phases as well during the 2 h after the removal of food. Moreover, to characterize the neurochemical mechanisms respon-

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sible for the modulation of ACh output during ingestive behavior, we investigated the effect of a nonsedative dose of abecarnil, a β-carboline derivative with potent anxiolytic activity (31,32), on the changes in ACh output during fasting, feeding, and satiation.

Finally, because neurotransmission mediated by γ-aminobutyric acid type A ($GABA_A$) receptors plays an important role in the regulation of emotional processes and ingestive behaviors (3,4,29) and exerts a tonic modulatory action on the activity of the septohippocampal cholinergic fibers (2,18), we also evaluated the functional state of central $GABA_A$ receptors by measuring *t*-[$^{35}$S]butylbicyclophosphorothionate ([$^{35}$S]TBPS) binding in the cerebral cortex, hippocampus, and septum of rats after fasting and feeding.

## METHOD

### *Animals and Diet Regimen*

Male Sprague–Dawley CD rats (Charles River, Como, Italy), with body masses of 180 to 220 g at the beginning of experiments, were housed in groups of three in wire-bottom cages (wire mesh, 7 by 12 inches). They were exposed to a 12L:12D cycle (lights on at 0800h), a constant room temperature of 23 ± 2°C, and 65% humidity. After arrival at the animal facility, rats were acclimatized for a minimum of 7 days, during which time they had free access to food and water. From the second week, groups of rats were trained separately to consume their daily food intake in a limited period of time. As previously described (6), the animals were allowed to consume their normal daily meal in 2 h; food (rat food pellets; Standard Diet GLP, Mucedola, Italy) was presented once a day at 1100 h and removed at 1300 h, whereas water was provided ad lib. Rats showed a marked decrease in their rate of growth during the first week of training, but this parameter returned to normal values after 15 days. Experiments were performed after 5 weeks of training, by which time each rat ate a relatively constant amount of food on successive days. At the end of each feeding session, the amount of food consumed was determined from the difference between the weight of food at the start of the session and that at the end. Two different groups of rats were used for microdialysis and [$^{35}$S]TBPS binding experiments.

All animal experimentations have been conducted in accordance with the statement revised and approved by the Society for Neuroscience in January 1995 and with the guidelines for care and use of experimental animals of the European Economic Community (86/609; D.L.:27.01.1992, No. 116).

### *Microdialysis Studies*

Four hours after the 33rd training session, each rat was implanted with a microdialysis probe. The animals were anesthetized with chloral hydrate (0.4 g per kilogram of body mass, IP), and dialysis tube with a wet-tube outer diameter of 320 μm (AN 69-HF; Hospal-Dasco, Bologna, Italy) was implanted at the level of the dorsal hippocampus or prefrontal cortex, according to the Paxinos atlas (A −3 from the bregma, V −3 from the dura for the hippocampus; A +4 from the bregma, V −1.5 from the dura for the prefrontal cortex). The active dialysing portions of the probes were 10 and 8 mm for the hippocampus and prefrontal cortex, respectively. Surgery was performed in both areas according to the transversal microdialysis technique described previously (17). Ringer's solution [3.0 mM KCl, 125 mM NaCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23 mM $NaHCO_3$, and 1.5 mM potassium phosphate (pH 7.3)] was pumped through the dialysis probe at a constant rate of 2 μl/min.

After surgery, rats were housed individually and perfused for at least 3 h. An additional training session was performed the next day (day 34) between 1100 and 1300 h as usual. During this session the probes were perfused for 4 h. The behavioral test and concurrent collection of microdialysis samples were performed on day 35. After a 1-h settling period, samples were collected every 20 min for the 2 h preceding food presentation, during food intake, and for the 2 h after food removal. The effect of abecarnil was investigated by administering the drug intraperitoneally in a volume of 0.3 ml per 100 g of body mass 40 min before food presentation.

Microdialysis samples (40 μl) were analyzed for ACh by high-performance liquid chromatography with electrochemical detection as described (12). Neostigmine (0.1 μM) was added to the Ringer's solution to achieve detectable amounts of ACh in the dialysate. The detection limit for ACh was 0.05 pmol per injection. The average concentration of ACh in the last three samples before the 2 h preceding food presentation was taken as 100% and all subsequent values were expressed as means ± SEM of the percentage variation from basal values.

We have consistently observed that rats implanted with a microdialysis probe show a slight decrease in their food intake.

### *[$^{35}$S]TBPS Binding*

At the end of the fifth week of training, rats were divided into three groups: those in the first group were killed 20 min before the next scheduled presentation of food, whereas those in the second and third groups were killed 1 and 2 h after food presentation, respectively; thus, the animals in the third group were killed immediately after food removal. The cerebral cortex, hippocampus, and septum were rapidly dissected out, and [$^{35}$S]TBPS binding was assayed as previously described (10,30). The fresh brain tissue, which is rich in GABA content and other modulators of $GABA_A$ receptor function, was homogenized with a Polytron PT 10 (setting 5 for 20 s) in 50 vol of ice-cold 50 mM Tris-citrate buffer (pH 7.4 at 25°C) containing 100 mM NaCl. The homogenate was centrifuged at 20,000 × *g* for 20 min, and the resulting pellet was reconstituted in 50 vol of Tris-citrate buffer for the binding assay. [$^{35}$S]TBPS binding was determined in a final volume of 500 μl, consisting of membrane preparation [200 μl (200 to 300 μg of protein) for cerebral cortex and hippocampus; 150 μl (150 to 200 μg of protein) for septum], 50 μl of 2 nM [$^{35}$S]TBPS, 50 μl of 2M NaCl, and 200 or 250 μl of Tris-citrate buffer. The reaction was initiated at 25°C by the addition of membranes and terminated 90 min later by rapid filtration through glass-fiber filters (GF/B;Whatman, Clifton, NJ) in a filtration manifold (model M-24, Brandel). The filters were rinsed twice with 4 ml of ice-cold Tris-citrate buffer and then dissolved in 3 ml of scintillation fluid. Filter-bound radioactivity was quantitated with a liquid scintillation spectrometer (Packard). Non specific binding was defined as binding in the presence of 100 μM picrotoxin, and represented ~10% of total binding. Protein concentration was assayed by the method of Lowry et al. (22) with bovine serum albumin as standard.

### *Motility Measures*

To study the locomotor activity we placed the animals in a new cage (three per cage), and measured exploratory behav-

iour 90 and 20 min before food presentation, with an Omnitech behaviour meter (Omnitch Electronics, OH). The meter measured horizontal activity by indicating the total number of interruptions of the horizontal beam sensor during the 10-min test period.

*Drugs and Chemicals*

Abecarnil (isopropyl-6-benzyloxy-4-methoxymethyl-β-carboline-3-carboxylate) (Schering A.G., Berlin, Germany) was dissolved in distilled water with 50 μl of Tween 80 (Aldrich Chemicals, Milwaukee, WI) per 5 ml. [$^{35}$S]TBPS (specific activity, 70–100 Ci/mmol) and scintillation fluid (Atomlight) was purchased from New England Nuclear. Other chemicals were obtained from commercial sources.

*Statistical Analysis*

Biochemical data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's test. For microdialysis studies, between-groups comparisons were performed by one-way or two-ways ANOVA for repeated measures, and post hoc comparisons were performed by Newman–Keuls test. A $p$-value of $<0.05$ was considered statistically significant.

## RESULTS

*Food Consumption*

Figure 1 shows that the amount of food consumed per rat during the 2-h feeding period increased for the first 3 weeks of training and then remained steady over the last 2 weeks. Food intake increased rapidly after the first 2 or 3 days of the training regimen. Moreover, the amount of food consumed per rat in 2 h was similar to that for rats trained to eat for 2 h under a reversed light–dark cycle with an equal diet regimen (data not shown). Trained rats exhibited increased motor activity during the 20 to 30 min preceding food presentation (120 ± 15 and 174 ± 21 horizontal beam interruption/10 min, 90 and 20 min before food presentation, respectively).

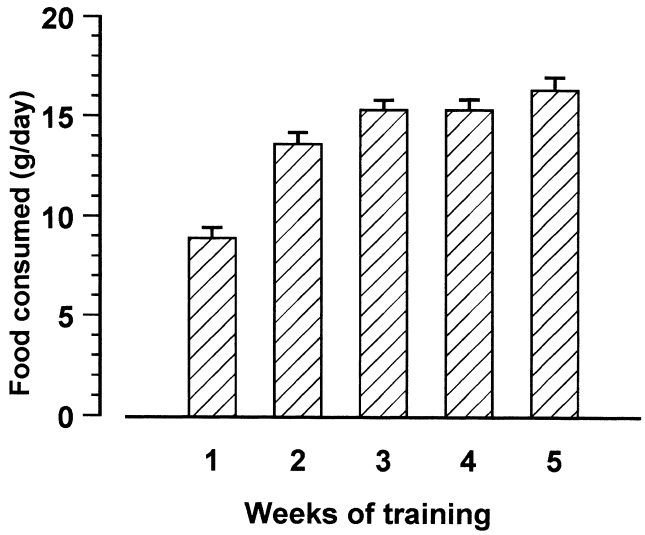


FIG. 1. Daily food consumption per rat during each of the 5 weeks of training. Animals were allowed access to food once a day for 2 h (1100 to 1300 h) during the light phase. Data are means + SEM for 40 rats.

*Microdialysis Studies*

Baseline values of ACh output during the 2 h preceding food presentation were 1.6 ± 0.12 pmol in the prefrontal cortex and 3.8 ± 0.25 pmol in the hippocampus (means ± SEM, $n$ = 35). The release of ACh was increased, although not significantly, 20 min before food presentation in both the prefrontal cortex and hippocampus (Fig. 2). This increase became significant in both brain areas (+49% in prefrontal cortex, +55% in hippocampus) 20 min later, immediately before food presentation. During the first 20 min of food intake, ACh output increased further to 198 and 175% of basal values for the prefrontal cortex and hippocampus, respectively. In the cortex, ACh release was maximal (+220%) after 40 min of food intake, remained significantly increased during the remainder of the consummatory phase, and returned to baseline values 60 min after food removal. One-way ANOVA revealed a significant effect of food intake on ACh release, $F(17, 71) = 314.687$, $p < 0.001$. In the hippocampus, ACh release was maximal after 20 min of feeding, remained significantly increased throughout the remainder of the consummatory phase, and the returned to baseline values 40 min after food withdrawal, $F(17, 71) = 81.611$, $p < 0.001$. In the absence of food intake the increase in ACh release in the anticipatory phase was still present, lasted not more than 40 min, returning to basal values in 60 min, $F(17, 71) = 5.662$, $p < 0.001$ (for the prefrontal cortex); $F(17, 71) = 67.144$, $p < 0.001$ (for the hippocampus). Moreover, control rats (injected with vehicle) showed an increase in ACh release both in the prefrontal cortex, $F(17, 71) = 77.999$, $p < 0.001$, and the hippocampus, $F(17, 71) = 78.407$, $p < 0.001$, similar to that observed in untreated rats (Fig. 2).

Given that high doses (1 to 3 mg/kg) of abecarnil enhance food consumption and reduce motor activity in rats and cats (9,11,26,28), we therefore evaluated the effects of a low dose (0.1 mg/kg, IP) of this agent administered 40 min before food presentation on food intake and motor behavior in fasted rats. Consistent with previous results (9), this dose of abecarnil had no significant effect on motor activity or food intake in trained rats (motor activity was 129 ± 18 vs. 144 ± 23 horizontal beam interruption/10 min; food intake was 16.2 ± 0.8 g vs. 16.4 ± 0.6 g for controls and abecarnil-treated rats, respectively). As expected (14), abecarnil at this dose reduced ACh output 40 min after its administration by 42 and 18% in the prefrontal cortex, $F(17, 71) = 109.354$, $p < 0.001$, and hippocampus, $F(17, 71) = 19.720$, $p < 0.001$, respectively, thus preventing the increase in the extracellular concentration of ACh associated with the anticipatory phase (Fig. 3). In contrast, abecarnil did not prevent the stimulatory effect of food intake on ACh release. Although during the consummatory phase, the extracellular concentration of ACh in the prefrontal cortex did not achieve values significantly different from baseline values, the relative increase in this parameter during the first 20 min of food intake was similar in both brain areas (~50%) to that observed in control rats. Finally, in absence of food intake the effect of abecarnil on basal ACh release lasted for at least 180 min. Two-way ANOVA of the effect of food intake in the prefrontal cortex of abecarnil- or vehicle-treated rats revealed a significant main effect of treatment, $F(1, 143) = 23.592$, $p < 0.001$; a significant main effect of repeated measures, $F(17, 143) = 107.250$, $p < 0.001$; and a significant interaction between factors, $F(17, 143) = 40.509$, $p < 0.001$. Two-way ANOVA of the effect of food intake in the hippocampus of abecarnil- or vehicle-treated rats revealed a significant main effect of treatment, $F(1, 143) = 1000$, $p < 0.001$; a signif-

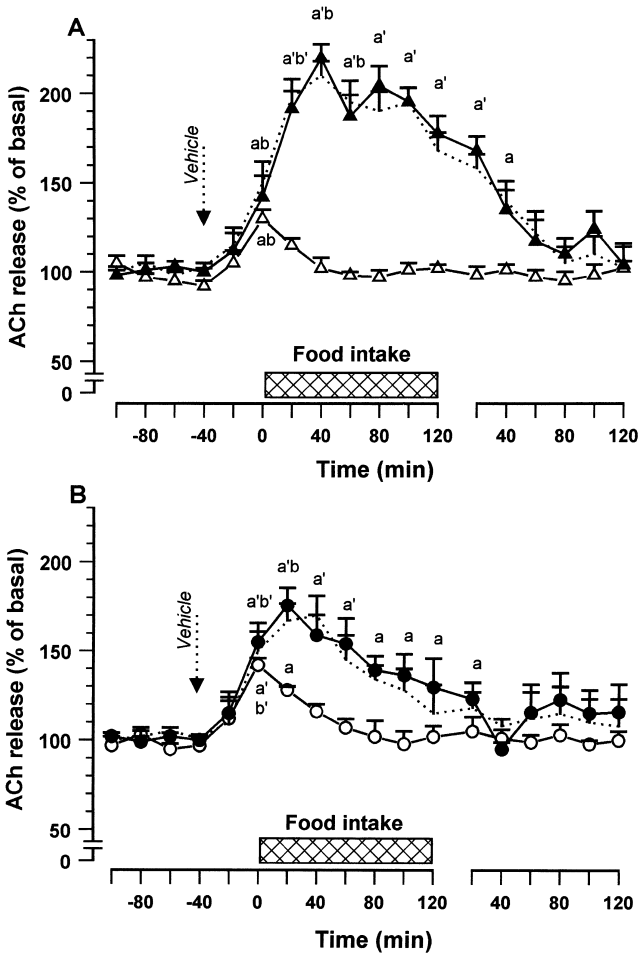


FIG. 2. Effect of anticipation and consumption of food on the extracellular concentration of ACh in the prefrontal cortex (A) and hippocampus (B) of fasted rats. ACh release was measured in freely moving rats during the 2 h preceding food presentation, the consummatory phase, and the 2 h after food intake in three different groups of rats: untreated rats (closed symbols); rats injected with vehicle 40 min before food presentation (no symbols, dotted line); rats untreated that had no access to food (open symbols). Data are means + SEM of at least 12 rats and are expressed as a percentage of basal values $^{a}p < 0.05$, $^{a}p < 0.01$ vs. basal values. $^{b}p < 0.05$, $^{b}p < 0.01$ vs. preceding time point.

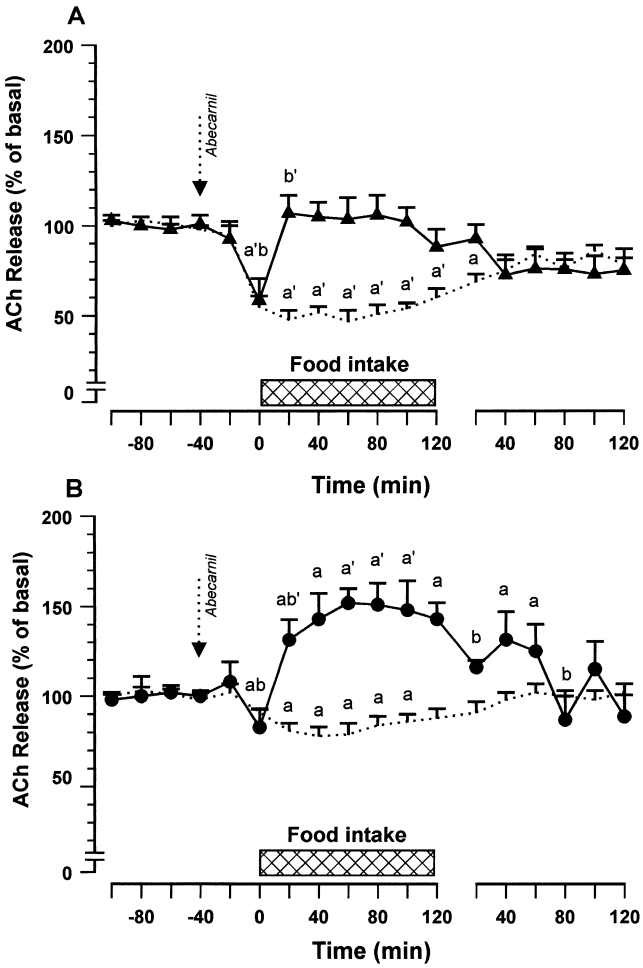


FIGURE 3. Effects of abercarnil on the increase in ACh release in the prefrontal cortex (A) and hippocampus (B) induced by the anticipation and consumption of food in fasted rats (closed symbols) or in absence of food intake (no symbols, dotted line). Abecarnil (0.1 mg/kg, IP) was administered 40 min before food presentation. Data are means + SEM of at least 12 rats and are expressed as a percentage of basal values. $^{a}p < 0.05$, $^{a}p < 0.01$ vs. basal values. $^{b}p < 0.05$, $^{b}p < 0.01$ vs. preceding time point.

icant main effect of repeated measures, $F(17, 143) = 114.151$, $p < 0.001$; and a significant interaction between factors, $F(17, 143) = 68.660$, $p < 0.001$.

### *[35S]TBPS Binding*

Animals killed immediately after the 2-h feeding period showed a significant decrease in [35S]TBPS binding to membranes prepared from the cerebral cortex, $F(2,44) = 78.652$, $p < 0.01$, hippocampus, $F(2,44) = 67.524$, $p < 0.001$, or septum, $F(2, 44) = 154.253$, $p < 0.001$, compared with rats killed 20 min before scheduled food presentation (Fig. 4). This effect of food intake on [35S]TBPS binding was apparent earlier (1h after food presentation) in the septum than in the other two brain areas.

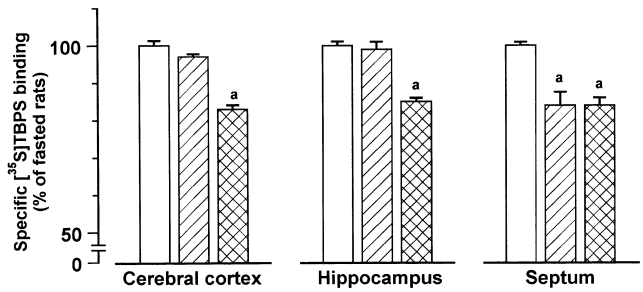


FIG. 4. [35S]TBPS binding to membranes prepared from the cerebral cortex, hippocampus, and septum of rats killed 20 min before (open bars), 1 h after (hatched bars), or 2 h after (crosshatched bars) food presentation. Data are means + SEM of 15 rats and are expressed as a percentage of the values obtained for the rats killed before food presentation. $^{a}p < 0.05$ vs. rats killed before food presentation.

## DISCUSSION

Our data demonstrate that, for rats habituated for 5 weeks to eat their daily meal during a fixed 2-h period, the extracellular concentration of ACh in the prefrontal cortex and hippocampus is increased during the anticipatory phase (20 to 40 min) preceding food presentation and further increased during the consummatory phase. These observations are consistent with the results of previous studies showing that cholinergic pathways to the frontal cortex and hippocampus are activated simultaneously by various stimuli, including arousal, motor activation, attention, as well as auditory and visual stimuli (1,13,21,25,27). Whereas the extent of the increase in ACh release during the anticipatory phase was similar in both brain areas (+49% in prefrontal cortex, +55% in hippocampus), food intake elicited a greater increase (+80%) in ACh output in the prefrontal cortex than in the hippocampus (+20%). The simultaneous increase in ACh release in both the prefrontal cortex and hippocampus during the anticipation of food presentation is consistent with the idea that cholinergic afferents to these brain areas are sensitive to changes in emotional state during the anticipation of reward presentation (20). This conclusion is further supported by the observation that the anxioselective benzodiazepine receptor ligand abecarnil (31,32) abolished the increase in ACh output in the prefrontal cortex and hippocampus during the anticipatory phase. Because abecarnil elicited this effect at a dose that exhibits an anticonflict action but fails to reduce either the state of arousal or motor activity (9,31,32), the anticipation-induced increase in ACh release is likely attributable to changes in the emotional state of the animals rather than to the motor activation induced by reward anticipation. A similar dissociation between locomotor activity and increases in ACh release in these two brain areas was recently described (1).

The observation that abecarnil failed to prevent the increase in ACh release elicited by food intake in both the prefrontal cortex and hippocampus suggests that emotional state does not play a major role in the regulation of cortical hippocampal cholinergic function during the consummatory phase. Moreover, these data may suggest that the changes in cholinergic function during the anticipatory phase are the consequences rather than the cause of the changes in emotional state. Thus, stimuli associated with the consummatory phase may increase central cholinergic activity without altering the emotional state of the animal. However, because behavioral experiments were not performed in our study, the functional relationship between ACh release and emotional state remains to be further clarified.

The fact that abecarnil prevented the enhancement of ACh release during the anticipatory phase but not that during the consummatory phase also supports the concept that at least two different neurochemical mechanisms participate in the regulation of cholinergic pathways to the frontal cortex and hippocampus: one associated with changes in emotional state, and another associated with different stimuli related to the consummatory phase. Because abecarnil is a selective positive allosteric modulator of $GABA_A$ receptors (31,32), a $GABA_A$ receptor-mediated mechanism might play an important role in modulating emotional state and the changes in cholinergic function during the anticipatory phase.

A role for GABAergic transmission in the changes in ACh output associated with food anticipation is also supported by the observation that [$^{35}$S]TBPS binding was markedly higher in the cerebral cortex, hippocampus, and septum of fasted rats killed 20 min before scheduled food presentation compared with the corresponding values for rats killed 1 or 2 h after food presentation. Given that the binding of [$^{35}$S]TBPS, which interacts selectively with the chloride channel associated with $GABA_A$ receptors, is increased by various stressful stimuli (5,10,33) as well as by drugs that both reduce $GABA_A$ receptor function and induce proconflict behavior in rats and anxiety attacks in monkeys and humans (5,7), $GABA_A$ receptor function may be reduced in the brains of fasted rats. Such a decrease in $GABA_A$ receptor function might, in turn, promote the activation of cholinergic neurons during the 20 to 40 min preceding the presentation of food. It is likely that this reduction in $GABA_A$ receptor function is the consequence of stressful stimuli elicited, at least in part, by starvation. To further clarify this point, experiments are in progress to evaluate whether the anticipation of food increases [$^{35}$S]TBPS binding in 22 h fasted rats when compared to rats receiving food ad lib.

The insensitivity to abecarnil of the mechanism responsible for the increase in ACh release during the consummatory phase suggests that GABAergic transmission does not play a major role in the enhancement of cholinergic function during food intake. Thus, the increase in ACh output during the consummatory phase is likely regulated by other neurochemical mechanisms known to be important in the regulation of ingestive behavior and food reward (3,4). However, a role for the abecarnil-insensitive motor component associated with food intake cannot be excluded.

Finally, our data also demonstrate that habituation to receiving the daily meal during a fixed 2-h period for 35 days does not reduce the increase in ACh release induced both by anticipation of food presentation and by food intake. Thus, rats do not appear to develop tolerance to the changes in emotional state associated with reward presentation or to other stimuli related to the consummatory phase.

## ACKNOWLEDGEMENTS

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