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10 July 1970

Acetylcholine Concentrations in Rat Brain: Diurnal Oscillation

Abstract. *A diurnal oscillation of acetylcholine concentrations in rat brain has been demonstrated by gas chromatography. Peak concentrations occur at 2 hours of light, and a trough is seen at 6 hours of darkness. This pattern is observed only in grouped rats, and emerges after at least 18 days of prior conditioning in an environment with controlled light, humidity, and temperature.*

In mammalian brain, the concentrations of some biogenic amines vary with a circadian periodicity (*1*). Also, light regulates the pineal activity of hydroxyindole-*O*-methyltransferase (*2*) and of 5-hydroxytryptophan decarboxylase (*3*), the enzymes responsible for synthesis of melatonin and serotonin, respectively. These results prompted us to study whether the brain concentrations of acetylcholine (ACh) exhibit a circadian rhythmicity. Such a periodicity is certainly important for interpreting experimental results of studies concerning long-term effects of drugs on brain ACh concentrations.

In confirmation of a report by Friedman and Walker (*4*), our results in rats show that brain ACh concentrations oscillate diurnally. However, whereas Friedman and Walker reported that the concentrations of ACh in the midbrain and caudate nucleus of rats peak at 4 hours of dark we found that the concentrations of ACh in the whole brain peak at 2 hours of light. These results were obtained with the use of a modification of an existing gas-chromatographic procedure for estimation of ACh in tissue extracts (*5*, *6*).

Sprague-Dawley, male, adult rats (Zivic Miller, Pittsburgh, Pennsylvania) (300 to 350 g) had unrestricted access to food and water, while housed either in groups of eight per cage or isolated (one animal per cage) in a soundproof room maintained at 23°C. The light cycle was controlled to provide 12 hours of light. The animals were decapitated at six evenly spaced times throughout a 24-hour cycle in groups of at least four rats each. Extreme care was taken to avoid causing undue stress or startling the animals. These experiments were all performed from October through February.

In our modified procedure we rapidly excised the brains (25 seconds) and homogenized them in 5 ml of ice-cold 0.4*N* perchloric acid with a Polytron ultraspeed homogenizer at full speed for 30 seconds. The brain weight was calculated by weighing the tubes before and after the brain was added. The final dilution of the homogenate was 4 ml of 0.4*N* perchloric acid per gram of wet brain. Brain homogenates were kept in ice for 30 minutes; they were then centrifuged in a Sorvall SS3 centrifuge in a cold room (2°C) at 9000*g* for 15 minutes.

A portion of the supernatant (3 ml) was transferred to another tube, and propionylcholine chloride (PrCh) (25 nmole; 25 μl in water) as an internal standard and 0.19 ml of potassium perchlorate (7.5*M*) in water were added. Propionylcholine chloride is a suitable internal standard because by a combination of gas chromatography and mass spectrometry it has been established that rat brain does not contain this compound (*7*). This mixture was centrifuged for 15 minutes at 9000*g* in the cold. Quaternary ammonium compounds in the supernatant (*p*H ranging between 4.20 and 4.45) were precipitated as the Reinecke salts in the presence of 50 m*M* tetraethylammonium chloride as coprecipitant. After the precipitates were freeze-dried, they were converted to their corresponding chlorides by batch treatment with Biorex 9 (Cl^-) (*5*) in methanol. The methanol was then transferred to another tube and dried prior to preparation for gas-chromatographic assay.

The esters were next demethylated with sodium benzenethiolate in methyl ethyl ketone and estimated by gas chromatography (*5*). Acetylcholine and PrCh are converted at this step to dimethylaminoethyl acetate and dimethylaminoethyl propionate, respectively, which, with the products of demethylation of other quaternary ammonium compounds, are extracted into chloroform (Fig. 1). Hexyldimethylamine was added as an external standard to each sample after demethylation with benzenethiolate was completed. This compound appears on the gas chromatogram with a retention time shorter than that of dimethylaminoethyl acetate (Fig. 1).

Acetylcholine concentrations reached a peak at 2 hours of light (L_2) and a trough at 6 hours of dark (D_6). In brains of rats kept in isolation (one animal per cage, 30 days or more) the ACh concentrations appeared statisti-

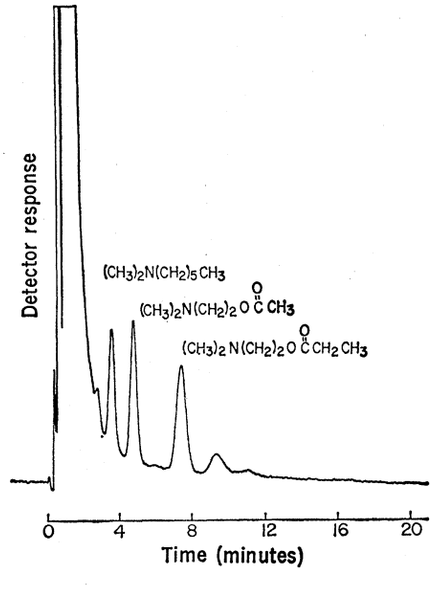

Fig. 1. Gas chromatogram of treated rat brain extract. Substances following solvent peak and identified by chemical formulas are, from left to right, hexyldimethylamine, external standard; dimethylaminoethyl acetate, demethylation product of endogenous acetylcholine; and dimethylaminoethyl propionate, demethylation product of propionylcholine, the internal standard. A silanized glass column (1.8 m; inside diameter, 2 mm) was packed with PAR 1 (80/120) coated with phenyldiethanolamine succinate (1 percent). The temperature of the column was 170°C; of the injection port, 240°C; and of the detector, 200°C. The flow rates were: carrier (N_2), 45 ml/min (80 psi); oxygen, 280 ml/min (20 psi); and hydrogen, 30 ml/min (26 psi). Dual flame ionization detectors were employed on a 5756 F&M gas chromatograph.

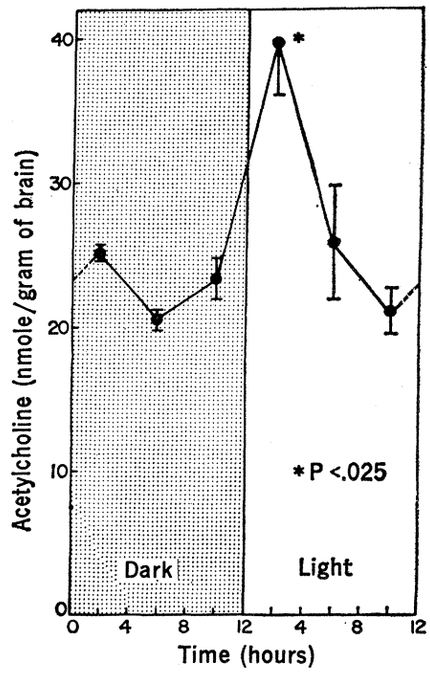

Fig. 2. Acetylcholine concentrations in brain of rats killed by guillotine throughout one 24-hour cycle. Each point represents the mean and standard error of at least three animals. Statistical difference was determined by Student's *t*-test. The *P* value was identical for any point on the curve with respect to the peak at L_2.

cally undistinguishable if measured either at D_6 (25.4 ± 0.2 nmole/g) ($n = 4$) or at L_2 (26.7 ± 2.5 nmole/g) ($n = 4$).

It appears from these results that the regulatory mechanism controlling ACh concentrations in rat brain does not maintain the steady state at a constant level throughout the day. Furthermore, this phenomenon occurs only in grouped animals, while in isolation the steady-state level of ACh is apparently maintained throughout the day.

This pattern was observed only in rats killed by decapitation with a guillotine. When animals were killed by total body immersion in liquid nitrogen, no diurnal pattern was observed. In fact, concentrations of brain ACh were identical at D_6 and L_2 and both were significantly lower than in rats killed by guillotine at the identical time period. However, freezing the animal in liquid nitrogen before extracting its brain for ACh assay may not reflect concentrations of rat brain ACh in vivo.

We have confirmed elsewhere (*8*) that brain ACh concentrations in rats killed by freezing in liquid nitrogen may reflect physical changes caused by the process of freezing and thawing on tissue compartmentation, leading to increased hydrolysis of the ester by acetylcholinesterase (*9*).

The diurnal pattern for brain ACh does not emerge in a group of rats housed under controlled conditions of light and temperature until a finite time has elapsed. Rats were placed in the controlled environment in groups of eight per cage immediately upon their arrival. Brain ACh concentrations were determined at 2 hours of light and 6 hours of darkness after 6, 13, 18, and 58 days of conditioning. Four animals were used at each time tested. Ratios of ACh concentrations at L_2 with respect to D_6 were calculated to be 0.94, 0.79, 1.79, and 1.93, respectively. These data indicate that at least 18 days are required before the concentrations of brain ACh are consistently higher at L_2 than they are at D_6. This probably reflects a synchronization of the diurnal pattern in animals housed under control conditions, as opposed to a random pattern prevalent in a group of rats which have just been delivered from the supplier.

This observation emphasizes the generally accepted belief that, in order to obtain meaningful and consistent results in animal experimentation, the animals have to be preconditioned to the experimental environment for a finite number of days. At least 18 days are required in studies involving the rat brain cholinergic mechanism. The obvious biochemical implications of the observed diurnal variation in concentrations of rat brain ACh provide a tool for gathering insight into the working hypothesis presented in this report. This diurnal rhythmicity could be used to detect physiological mechanisms regulating steady-state concentrations and turnover rate of brain ACh content.

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Brain Enhancement in Tadpoles: Increased DNA Concentration after Somatotrophin or Prolactin

Abstract. *Frog tadpoles, injected with prolactin or somatotrophin during early stages when the brain cell population is rapidly increasing, exhibited marked increases over sham-injected controls in body weight, brain weight, and brain DNA, throughout subsequent development. Animals treated with somatotrophin attained the increase in brain DNA during the infection period. Prolactin had little effect during this period, but brain DNA accumulation continued at an accelerated rate over the next 15 days, when the rate of DNA increment normally declined. Patterns of incorporation of tritiated thymidine confirmed that both hormones increased DNA accumulation, suggesting increased cell proliferation rather than decreased cell death.*

Mammalian prolactin and mammalian somatotrophin both promote general body growth in anuran tadpoles (*1*). The actions of the two hormones on the growth and development of the brain, however, are only vaguely understood. Early experiments, in which crude pituitary extract was adminis-