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Comparison of Two Methods for Estimating the Acetylcholine Turnover in Discrete Rat Brain Structures

R. BLUTH, R. LANGNICKEL, R. MORGENSTERN AND W. OELSZNER

Institute of Pharmacology and Toxicology, Humboldt-University Berlin, German Democratic Republic, 1040 Berlin, P.O.B. 140

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BLUTH, R., R. LANGNICKEL, R. MORGENSTERN AND W. OELSZNER. *Comparison of two methods for estimating the acetylcholine turnover in discrete rat brain structures.* PHARMACOL BIOCHEM BEHAV 20(2) 169–174, 1984.—The acetylcholine turnover rate was determined in olfactory tubercle, nucleus accumbens and striatum of rat brain. The calculation of turnover rates was carried out by means of two different methods: a two compartment analysis and the finite differences method. After pulse injection of [^{3}H]choline the radioactivity of both [^{3}H]acetylcholine and [^{3}H]choline was measured in the above mentioned brain areas. The contents of acetylcholine and choline were measured radioenzymatically. By using the two compartment model the following acetylcholine turnover rates were obtained: olfactory tubercle, 0.577; nucleus accumbens, 0.679; striatum, 1.110 (μmoles/g×hr). When using the finite differences method the values were: olfactory tubercle, 0.517; nucleus accumbens, 0.822; striatum, 1.115 (μmoles/g×hr). This demonstrates that the results obtained by applying the two different methods are nearly identical. Advantages and disadvantages of the two methods are discussed.

Acetylcholine Turnover Methods Striatum Mesolimbic areas

VARIOUS methods have been developed to determine the acetylcholine turnover in brain structures. To carry out most of these procedures labeled precursors are given either by intravenous pulse injection [17, 22, 30] or by infusion at constant rate [20,40], and it is postulated that steady state conditions exist during the experiments [7, 15, 23]. By measuring the content of acetylcholine and choline as well as the labeling in the two pools the acetylcholine turnover rate [23,30] can be calculated.

The analysis of the kinetics of labeled choline (and acetylcholine) led to the assumption that a two compartment model consisting of a choline and an acetylcholine pool is sufficient to describe the time course of the concentrations of choline and acetylcholine in the pools [1]. Recently, we were able to demonstrate that the compartment analysis can provide reliable results if the rate constants calculated for the acetylcholine pool and the choline concentration at zero time are used [1].

A second method widely used is the finite differences method. It is based on a calculated fractional rate constant for acetylcholine efflux and the acetylcholine concentration in brain tissue [23]. This method seems to be less time consuming and to have some other economical advantages over the compartment analysis. The present paper is to compare these two methods on the basis of experimental results obtained for the following forebrain structures: striatum, nucleus accumbens and olfactory tubercle, all of them being involved in the action of antipsychotic drugs [4].

MATERIALS

Experiments were carried out on male Wistar rats weighing 145–155 g (VEB Versuchstierproduktion Schönwalde). The animals received standard food and water ad lib. The following substances were used: Acetyl coenzyme A (Boehringer, Mannheim), heptanone (3) (Merck), cellulose MN 300 (Macherey, Nagel and Co.) and sodium tetraphenylboron (Jenapharm). [Methyl-^{3}H]choline chloride (77 Ci/mmole) and [acetyl-^{3}H]acetyl coenzyme A (500 mCi/mmole) were purchased from New England Nuclear.

METHOD

Determination of Acetylcholine and Choline Content

The rats were fixed in a special animal holder and sacrificed by a high intensity microwave beam (2 kW, 2450 MHz, 3 sec) focussed directly onto the skull since we found in separate experiments [2] that striatal acetylcholinesterase was completely inactivated after less than 3 seconds of this irradiation. These data are in agreement with those of Guidotti *et al.* [11] and Schmidt [29] in rats, and these times are clearly longer than those needed in mice [22]. Subsequently the brains were rapidly removed from the skull and dissected free-hand on ice after removing the dura and the surface blood vessels. Olfactory tubercle, nucleus accumbens and striatum were used in the experiments. The dissection of the brain regions is greatly facilitated after microwave fixation which provides an easy separation of the regions

TABLE 1

EQUATIONS OF TWO DIFFERENT METHODS FOR DETERMINING THE ACETYLCHOLINE TURNOVER RATE

Two Compartment Analysis Performed from Compartment b		Finite Differences Method
(1) q_b/q_{a_0}	$= K_1 \cdot e^{k_1 t} + K_2 \cdot e^{k_2 t}$	$k_{ACh} = \dfrac{\dfrac{SA_{ACh_{t_2}} - SA_{ACh_{t_1}}}{t_2 - t_1}}{\dfrac{(SA_{Ch} - SA_{ACh})_{t_1} + (SA_{Ch} - SA_{ACh})_{t_2}}{2}}$
(2) K_1	$= K_{ba}/(k_1 - k_2)$	
(3) $k_{aa} + k_{bb}$	$= -(k_1 + k_2)$	
(4) $k_{bb} \cdot k_{ba} - k_{aa} \cdot k_{bb}$	$= k_1 \cdot k_2$	
(5) $k_{oa} + k_{ba}$	$= k_{aa}$	
(6) k_{ab}	$= k_{bb}$	

q_b=Quantity of tracer in pool b, q_{a_0}=quantity of tracer in pool a at zero time. (Note: equation (1) is normalized and describes the relative quantity of tracer in pool b). K_1 and K_2=first and second coefficient of the exponential function, k_1 and k_2=slope of the exponential function. k_{ab}, k_{ba}, k_{oa}, k_{aa} and k_{bb} are rate constants. The latter two are the overall turnover rate constants for pool a and b, respectively. k_{ab} and k_{ba} represent the rate constants from pool b to pool a and reversely. k_{oa} is the rate constant for the efflux from compartment a. Equations (3) and (4) result in a quadratic equation with two real solutions for k_{aa} and k_{bb}.

k_{ACh}=Fractional rate constant for acetylcholine efflux.

SA_{ACh}=Specific activity of acetylcholine (DPM)/nmoles acetylcholine).

Sa_{Ch}=Specific activity of choline (DPM)/nmoles choline).

along their natural boundaries. The extraction of acetylcholine and choline from brain tissue was performed as described by Toru and Aprison [34]with slight modifications [2]. The volume of the extraction medium was 1.5 ml. All samples were run in duplicate. The separation of acetylcholine and choline was carried out by thin-layer-chromatography on cellulose plates. The migration solvent consisted of n-butanol/methanol/ethyl acetate/acetic acid/water (4/2/4/1/3). The acetylcholine and choline spots were localized indirectly by running two acetylcholine/choline standards per plate stained with Dragendorff-reagent. The acetylcholine was hydrolyzed to choline in an alkaline medium. The choline obtained in this procedure as well as the native choline were converted to labeled acetylcholine by choline acetyltransferase [27]. The incubation medium contained 50 nmoles acetyl coenzyme A, 0.3 mM eserine, 0.1 M sodium phosphate buffer, pH 7.4, 1 μCi/acetyl-^{3}H/acetyl coenzyme A and 50 μl standardized choline acetyltransferase preparation (254 pmoles/mg protein × min). The samples were incubated at 37°C for 45 minutes. After stopping the reaction in an ice water bath the extraction of [^{3}H]acetylcholine was performed by sodium tetraphenylboron dissolved in heptanone (3) (10 mg/ml) as described by Fonnum [10]. The organic phases were washed with sodium phosphate buffer, pH 7.4, and transferred into scintillation fluid consisting of dioxane, naphthalene, 2,5-Diphenyloxazole and 2,2'-p-Phenylene-bis-(5-phenyl-oxazole). The radioactivity was measured in a liquid scintillation counter (LKB, Ultrobeta 1210). The contents of acetylcholine and choline were calculated by using standard curves. For a detailed description of the whole procedure see [2].

Determination of Acetylcholine Turnover Rates

The rats received an intravenous (tail vein) pulse injection of [^{3}H]choline (100 μCi/100 g body weight). The quantity of the precursor administered was 13 nmoles choline/kg body weight. It was shown by other authors [24] that choline up to a dose of as high as 1 μmole choline/kg body weight × min did not change the steady state concentrations of endogeneous choline and acetylcholine. For the finite differences method the rats were sacrificed by the same microwave irradiation procedure as above 30 or 60 seconds after the administration of the labeled precursor. To calculate the acetylcholine turnover rate according to the two compartment model the animals were killed at various fixed times over a period of 10 minutes after the administration of the precursor. The brains were rapidly removed from the skull and olfactory tubercle, nucleus accumbens and striatum were prepared for the experiments.

Further experimental steps, the extraction of choline and acetylcholine from brain tissue and the separation of acetylcholine and choline, were performed such as above. The [^{3}H]choline and [^{3}H]acetylcholine were measured by liquid scintillation counting. The kinetic data or the contents of choline and acetylcholine were determined in separate experiments as it was described by Bohman *et al.* [3] and Schuberth *et al.* [30].

The level of labeled choline in blood was determined for a correction of the [^{3}H]choline content in brain tissue taking as a basis 3% blood content in brain [18]. Fitting the curves according to an exponential polynomial function (see Table 2) was performed on a Hewlett-Packard 9825 A desk top computer by using a Gauss-Newton iterative procedure. The calculated parameters of this function provide the calculation of the parameters of a compartment model. The mathematical equations [31] for calculating the acetylcholine turnover rates according to an open two compartment model from pool b are given in Table 1 (left side). The tracer [^{3}H]choline labels the pool a (choline) which is directly connected with pool b (acetylcholine). The description of the finite differences method is given in Table 1 (right side) [21].

RESULTS

The contents of acetylcholine and choline in olfactory tubercle, nucleus accumbens and striatum are given in Fig. 1. In all brain structures examined the levels of acetylcholine are about twice as high as those of choline. The highest level of acetylcholine was found in olfactory tubercle whereas no significant differences were seen between nucleus accumbens and striatum. On the other hand, the choline contents of the three brain structures did not differ.

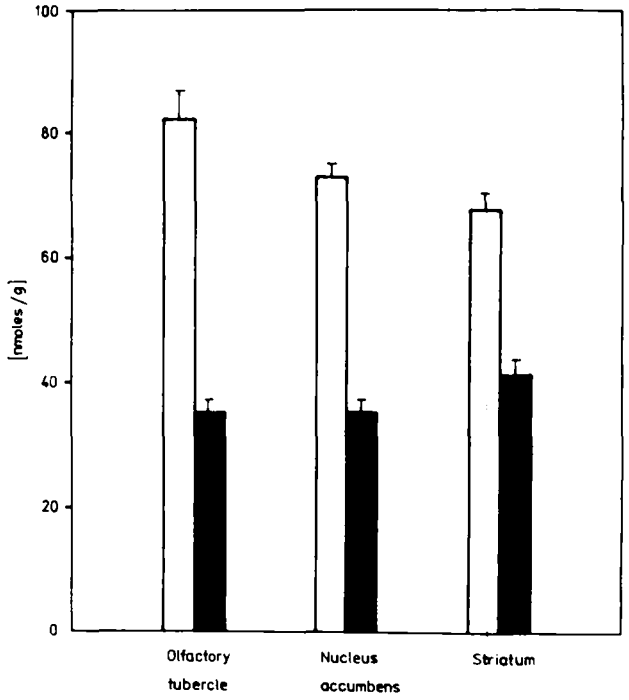

FIG. 1. Acetylcholine (open columns) and choline contents (black columns) in olfactory tubercle (n=22), nucleus accumbens (n=17) and striatum (n=28), mean value ± standard error of the mean. The acetylcholine content in olfactory tubercle is significantly higher than in nucleus accumbens ($p<0.025$) and striatum ($p<0.005$) (Mann-Whitney).

TABLE 2

CALCULATED PARAMETERS OF THE FUNCTION $y=K_1 \cdot e^{k_1 t} + K_2 \cdot e^{k_2 t}$ FOR DETERMINING THE RATE CONSTANTS OF THE TWO COMPARTMENT MODEL IN STRIATUM

Parameters of the Exponential Functions		Rate Constants of the Two Compartment Model Calculated From Compartment b
Choline	Acetylcholine	
$K_1=63752$	$K=37055$	$k_{ba}=0.44$
$K_2=36054$		$k_{ab}=0.78$
$k_1=-4.78$	$k_1=-1.29$	$k_{oa}=0.16$
$k_2=-0.19$	$k_2=-0.10$	
$R=0.87$	$R=0.74$	
$n=26$	$n=36$	

The kinetics of acetylcholine differs from that of choline with respect to the constants K_1 and K_2. Considering the acetylcholine kinetics: $-K_1=K_2=K$; K_1 is always negative.

After pulse injection of [^{3}H]choline a rapid labeling of brain structures occurred within 20 seconds which was followed by an exponential decrease of [^{3}H]choline. As recently shown [1] this decrease can be fitted best by using a second degree exponential function. The time course of specific labeling in the acetylcholine pool can be described by using the same function. However, the kinetic data of the two functions differ markedly.

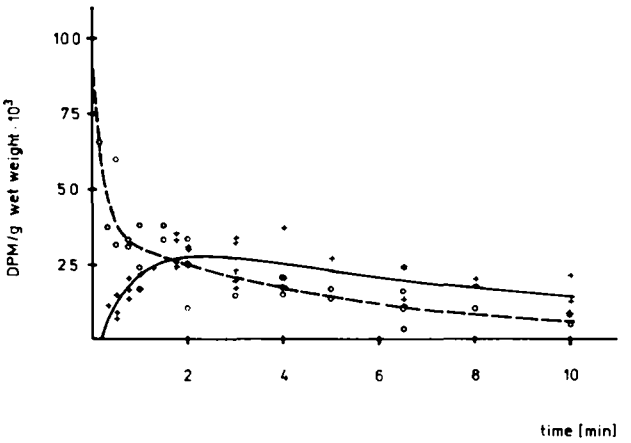

FIG. 2. Time course of [^{3}H]choline (O— — —O, n=26) and [^{3}H]acetylcholine (+ —— +, n=36) in striatum after pulse injection of [^{3}H]choline. The calculated parameters of the fitted functions are given in Table 2.

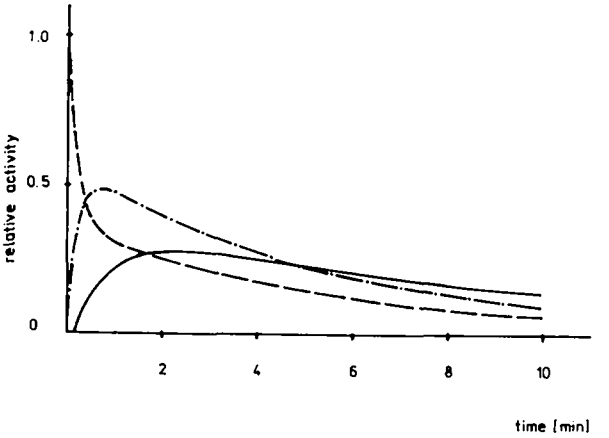

FIG. 3. Compartment analysis, only performed by using the data of [^{3}H]choline (— — —) in comparison with the curve of the measured [^{3}H]acetylcholine (——). Note the striking difference between the hypothetical [^{3}H]acetylcholine curve (— · —) and the [^{3}H]acetylcholine curve directly calculated from experimental data.

According to Shipley and Clark [31] the compartment analysis was made by using the kinetic data of the time course of labeled acetylcholine and by using the calculated labeled choline level at zero time as q_{a_0} (see Table 1). As an example the fitted functions for striatum are shown in Fig. 2. It will be mentioned that the intercept of the calculated [^{3}H]acetylcholine curve differs slightly from zero time. This difference may represent various metabolic steps and diffusion processes. The intercept of the iterated [^{3}H]acetylcholine curve with the x-axis was used as zero time ($t_{0_{ACh}}$) in the compartment analysis since this calculated zero time ($t_{0_{ACh}}$) is rather directly connected with the acetylcholine kinetic data than a zero time which is given by the time of the pulse injection of choline. To do this, the [^{3}H]acetylcholine curve was shifted to the left to originate from zero point of the coordinate system. The data of the [^{3}H]choline curve, the shifted [^{3}H]acetylcholine curve and the rate constants according to the two compartment model

TABLE 3

ACETYLCHOLINE TURNOVER RATES (TR_{ACh}) DETERMINED BY TWO DIFFERENT METHODS

Brain Structure	Two Compartment Model		Finite Differences Method	
Olfactory Tubercle	k_{ba}	=0.2709	k_{ACh}	=0.1044
	TR_{ACh}	=0.574 ± 0.033	TR_{ACh}	=0.517 ± 0.028
Nucleus Accumbens	k_{ba}	=0.3205	k_{ACh}	=0.1878
	TR_{ACh}	=0.679 ± 0.038	TR_{ACh}	=0.822 ± 0.026
Striatum	k_{ba}	=0.4434	k_{ACh}	=0.2739
	TR_{ACh}	=1.110 ± 0.067	TR_{ACh}	=1.115 ± 0.043

Rate constants in min^{-1}, TR_{ACh} in μmoles/g × hr, mean ± standard error of the mean.

are given in Table 2. The procedure provides a k_{ba} which is about five times lower compared with a hypothetical k_{ba} value which can be calculated from the time course of labeled choline alone. The kinetic curves of this compartment analysis are shown in Fig. 3. The difference between the [^{3}H]acetylcholine curve calculated from [^{3}H]choline kinetics and the [^{3}H]acetylcholine curve calculated from measured data is obvious. Next, the acetylcholine turnover rate was calculated by multiplying the rate constant k_{ba} by the choline content in brain tissue (Table 3).

An acetylcholine turnover rate according to the finite differences method could be obtained directly by using the equation given in Table 1 (right side) and multiplying the k_{ACh} value by the acetylcholine content (Table 3).

Different acetylcholine turnover rates were found in the different brain structures, a higher one in striatum and lower ones in the two mesolimbic areas.

DISCUSSION

There is a considerable number of papers in the literature reporting on an estimation of the activity of cholinergic systems in brain. It was shown by several authors that the contents of acetylcholine and choline [8, 9, 12, 13, 19] or the acetylcholine turnover rate [15, 17, 20, 26, 28, 30, 33, 36] have been used to indicate the activity of central cholinergic systems.

To estimate the acetylcholine turnover rate it is required to determine the contents of acetylcholine and choline and the kinetic data of labeled acetylcholine in the acetylcholine pool. When comparing our data with those in literature which used the same killing procedure (microwave irradiation), then the contents of acetylcholine and choline in corresponding brain structures agree well [5, 6, 8, 13, 14, 16]. In preliminary experiments we have also found comparable data for hippocampus (unpublished results). However, different values were reported depending on different experimental conditions [9, 12, 19]. It should be mentioned that 3 seconds for inactivating the acetylcholine metabolizing enzymes in the rat brain is quite long compared with 0.25 seconds in mice [22], however, this microwave head focussed technique is the most rapid irreversible inactivation technique for brain enzymes available at present [11, 16, 22, 32].

This paper was to compare two different methods to determine the acetylcholine turnover rate—the use of a two compartment model or the finite differences method—whether the methods supply reliable results. Therefore, two distinct series of experiments were performed. Following the injection of the labeled precursor, in the compartment analysis experiments specific labeling of choline and acetylcholine pools was measured at various fixed times over a period lasting 10 minutes. To accomplish the finite differences method the labeling in acetylcholine and choline pools was measured at two fixed times (30 seconds and 60 seconds) after injection of the labeled precursor. The calculation of the turnover rate in the first procedure is based on kinetic data of both the increase and decrease of labeled acetylcholine. On the other hand, the finite differences method uses the first phase of kinetics (i.e., the increase of labeled acetylcholine) only. Despite these striking differences the turnover rates obtained by the two methods are in the same rank order when comparing the three brain structures examined. Moreover, the data obtained for each brain structure did not differ markedly. This would mean that both methods are of comparable usefulness for estimating the acetylcholine turnover rate in discrete brain structures.

However, the compartment analysis may not be performed by using kinetic data of labeled choline since it was clearly shown that the hypothetical [^{3}H]acetylcholine differs markedly from [^{3}H]acetylcholine directly measured in brain tissue (Fig. 3). This difference was found in all brain structures examined and, as a representative example, the kinetic data of striatum calculated from [^{3}H]choline or [^{3}H]acetylcholine are given. This difference may represent the fact that a substantial portion of [^{3}H]choline given by pulse injection is rather associated with lipid metabolism than with the acetylcholine turnover [16]. Therefore, the kinetic data of the measured [^{3}H]acetylcholine were used to calculate the acetylcholine turnover rate.

Next, under steady state conditions the following equation should be valid:

$$\frac{d[ACh]}{dt} = k_{ba} \times [Ch] - k_{ab} \times [ACh] = 0.$$

When applying this equation to the data measured or calculated for striatum the following results were obtained:

$$TR_{ACh} = k_{ba} \times [Ch] = 1.11\ \mu\text{moles/g} \times \text{hr}$$
$$TR_{ACh} = k_{ab} \times [ACh] = 3.18\ \mu\text{moles/g} \times \text{hr}$$

A similar result was obtained for the mesolimbic structures too. This means that quite different acetylcholine turn-

over rates can be calculated only dependent on whether the level of choline or acetylcholine is used in the calculations. This striking discrepancy cannot be neglected and it has some consequences. First, only a portion of the acetylcholine determined in the experiments is directly involved in the actual acetylcholine turnover. Second, the extent of the discrepancy may somehow reflect the degree of compartmentation within the acetylcholine pool. And last, a calculation of acetylcholine turnover rate has to use the kinetic data of [^{3}H]acetylcholine and the choline level rather than the level of endogeneous acetylcholine. Thus, the compartment analysis provides a more detailed discussion of mechanisms underlying drug induced changes of acetycholine turnover rate. However, the procedure is very time consuming.

It is an advantage of the finite differences method to provide also correct results when information is required on the acetylcholine turnover rate in discrete brain areas. The method is appropriate to obtain many results within a relatively short time since it is less time consuming. As shown by Vocci *et al.* [37] reliable results can be obtained if the data determined in the experiments represent the steepest part of the acetylcholine formation curve. Since drug treatment can shift this curve to the left or to the right the equation of Zilversmit [39] and Neff *et al.* [21] can be used for comparisons between animals treated with different drugs [17].

When comparing our data with those in literature one can say that they are in good agreement with the many data reported on striatum [23, 25, 26, 35, 36, 38] and with the few ones reported on nucleus accumbens [26,40]. Some of them have been based on the finite differences method [23,35] and most of them have been based on a compartment analysis using an open single compartment system [23, 25, 26, 36, 38, 40].

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