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Differential effect of anionic and cationic drugs on the synaptosome-associated acetylcholinesterase activity of dog brain

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The evoked effects of the negatively charged drugs phenobarbital and barbituric acid, the positively charged imipramine, perphenazine and trifluoperazine, and the neutral primidone, on the synaptosome-associated acetylcholinesterase activity were studied. A marked increase in the enzyme activity was exhibited in the presence of low concentrations (up to 3 mM) of phenobarbital, barbituric acid and primidone. Higher concentrations (up to 10 mM), however, led to a progressive inhibition of the enzyme activity. However, the activity of the enzyme was not affected by imipramine, but it was decreased by perphenazine and trifluoperazine. Arrhenius plots of acetylcholinesterase activity exhibited a break point at 23.4°C for the untreated (control) synaptosomes, which was shifted to around 16°C in the synaptosomes treated with the charged drugs. The allosteric inhibition by F^- of acetylcholinesterase was studied in control synaptosomes and in those treated with the charged drugs. Changes in the Hill coefficients in combination with changes in Arrhenius activation energy produced by the charged drugs would be expected if it is assumed that charged drugs 'fluidize' the synaptosomal plasma membranes.

Acetylcholinesterase (EC 3.1.1.7) is a biologically significant component of the plasma membranes, contributing to their integrity and to the permeability changes occurring during synaptic transmission and conduction (Grafius *et al.*, 1971). It is well known that general central-nervous-system depressants (e.g. barbiturates) produce functional tolerance and physical dependence after repeated administration (Harvey, 1980). Therefore, from a mechanistic standpoint, alterations in membrane function induced by these drugs would be expected to correlate with the central depressant actions. Barbiturates can increase the fluidity, lipid disorder or entropy of synaptosomal plasma membranes, thus causing changes in membrane-bound enzyme activities (Pastuszko, 1980; Deliconstantinos, 1983).

We have studied the effect of small amphipathic compounds (e.g. cholesterol and docecanol or their glucosides, steroid hormones, negatively charged phospholipids etc.) on the activity of some plasma-membrane-bound enzymes, suggesting that these compounds can alter the membrane fluidity, causing functional changes in the allosteric properties of integral enzymes (Alivisatos *et al.*, 1981*a*,*b*; Alivisatos & Deliconstantinos, 1982; Deliconstantinos & Ramantanis, 1983; Deliconstantinos *et al.*, 1983; Tsakiris & Deliconstantinos, 1984; Deliconstantinos, 1984).

In the present work we studied the effect of the negatively charged barbiturates (phenobarbital, barbituric acid), the neutral primidone and the positively charged antidepressants imipramine, perphenazine and trifluoperazine, on the acetylcholinesterase activity of dog brain synaptosomes. Synaptosome-associated acetylcholinesterase activity was used in the present study as a marker of membrane changes in conditions where charged drugs may perturb the membrane fluidity, since the fluid state of the lipids regulates membrane-associated enzymes.

Materials and methods

Dog brain synaptosomes were prepared and qualitatively assessed as previously described (Papaphilis & Deliconstantinos, 1980). Phenobarbital and barbituric acid were made up as 30 mM stock solution, and the pH was adjusted to 8.0 before dilution into the assay. Imipramine, perphenazine and trifluoperazine were made up as 30 mM stock solutions, and the pH was adjusted to 7.0 before dilution into the assay. Primidone was made up as a 8.5 mM stock solution, owing to the

limitations inherent in the insolubility of primidone in aqueous media, and the pH adjusted to 8.0 before dilution into the assay. The anionic forms of phenobarbital and barbituric acid predominate at pH 8.0, and the cationic forms for imipramine, perphenazine and trifluoperazine predominate at pH 7.0. Primidone is neutral at pH 8.0. Preincubations of the synaptosomes with the drugs were performed at pH 8.0 for phenobarbital, barbituric acid and primidone, and at pH 7.0 for imipramine, perphenazine and trifluoperazine. Preincubations of synaptosomes (0.5mg of protein/ml) with different concentrations of the drugs were performed for 1h at 30°C, in 35mM-Tris/HCl/0.3M-sucrose, in a final volume of 1.5ml with continuous magnetic stirring, and then the enzyme activity was determined.

Acetylcholinesterase activity was determined by measuring the hydrolysis of acetylthiocholine by the method of Ellman *et al.* (1961) at temperatures of 5–42°C at 2°C intervals. The assay mixture (3ml) contained 0.5mM-acetylthiocholine iodide, 0.125mM-5,5′-dithiobis-(2-nitrobenzoic acid) 120mM-NaCl, 0.24M-sucrose and 0.1M-Tris/HCl, pH 8.0, for the anionic drugs and for the neutral primidone, and pH 7.0 for the cationic drugs. Protein concentration was 0.1mg/3ml incubation mixture. The reaction was followed spectrophotometrically by measuring the increase in A_{412} with a Beckman Acta MNI spectrophotometer. Control experiments indicated that the drugs did not interfere with the enzymic determinations.

The synaptosomes were solubilized with 0.5% (w/v) Lubrol-PX at 4°C for 4h under magnetic stirring, in 0.24M-Tris/HCl/8.4% (w/v) sucrose, pH 7.4, containing 1.5mg of synaptosomal protein/ml. After this treatment the sample was centrifuged at 150000*g* for 1h, to obtain the Lubrol-soluble and -insoluble fractions as supernatant and pellet respectively. The supernatant contained 1.2mg of synaptosomal protein/ml. The protein content was determined by the method of Lowry, as described by Miller (1959), with bovine serum albumin (Sigma) as standard. The final concentration of the detergent in the assay mixture for measurement of acetylcholinesterase activity was less than 0.008%, which did not interfere with the enzymic determination.

For the assay of the inhibition of the acetylcholinesterase activity by fluoride (F^-), the reaction mixture contained increasing amounts of NaF, as indicated in Fig. 3. Lines were fitted to the data points in Arrhenius plots and in Hill plots by regression analysis.

Results

Changes in synaptosome-associated acetylcholinesterase activity at different concentrations of charged drugs are illustrated in Fig. 1. Anionic and neutral drugs exhibited an increase in enzyme activity (Fig. 1*a*). A dramatic increase (~100%) in the presence of phenobarbital and a marked increase (~70%) in the presence of barbituric acid

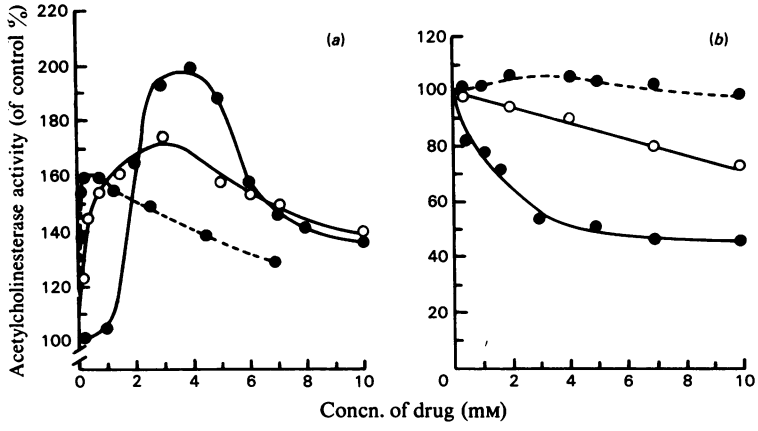

Fig. 1. *Effect of different concentrations of charged drugs on the synaptosome-associated acetylcholinesterase activity* (*a*) Effect of the anionic drugs phenobarbital (●——●) and barbituric acid (○——○) and of the neutral primidone (●---●) on the enzyme activity. (*b*) Effect of the cationic drugs imipramine (●---●), perphenazine (○——○) and trifluoperazine (●——●) on the enzyme activity. The value of the acetylcholinesterase activity estimated at 20°C and pH 8 was $0.58 \pm 0.02 \Delta A_{412}$/min per mg of protein, and at pH 7 it was $0.30 \pm 0.01 \Delta A_{412}$/min per mg. For experimental details see the Materials and methods section. Each point represents the average value of duplicate determinations from a typical experiment which has been repeated three times. The values of the coefficient of variation were within ±5% from the mean value.

was shown at concentrations around 3–4mM. A considerable increase in the enzyme activity (~60%) was also appeared at 0.3–0.8mM concentrations of the neutral primidone. Higher concentrations of the drugs, however, led to a progressive inhibition of the enzyme activity with respect to the maximal percentage of stimulation. On the other hand, positively charged drugs (Fig. 1*b*) exhibited inhibition or even no change in the enzyme activity. A considerable decrease (~55%) in the enzyme activity was shown by trifluoperazine, a slight decrease (~25%) by perphenazine and no change by imipramine at concentrations of 0.3–10mM.

When studying the effects *in vitro* of charged drugs on the synaptosome-associated acetylcholinesterase activity, it is important to know that they do not produce membrane destruction. Synaptosomes' structural integrity was indicated by similar low values of lactate dehydrogenase activity in normal and in drug-treated preparations.

Solubilization of the acetylcholinesterase with the non-ionic detergent Lubrol-PX resulted in an approx. 80% increase in the enzyme activity in the supernatant soluble fraction. The increased activity of acetylcholinesterase in the soluble fraction is probably the result of both an increase in the conformational flexibility of the membrane-bound enzyme, achieved by relief of a physical constraint imposed by the lipid bilayer on the protein molecule, and the release by membrane dissolution of the acetylcholinesterase that is trapped in the synaptosomal cytoplasm. The lack of effect of the charged drugs on the solubilized enzyme reveals that they do not affect the enzyme directly, but the evoked functional changes in the intact synaptosome-associated enzyme are probably mediated through changes in membrane fluidity.

The modulation of the membrane fluidity by the influence of the charged drugs was detected by determination of possible changes in transition temperatures of synaptosome-associated acetylcholinesterase, and by determination of the Hill coefficients for the inhibition of the enzyme activity by F^- (Deliconstantinos & Ramantanis, 1983; Deliconstantinos, 1983; Tsakiris & Deliconstantinos, 1984). The temperature-dependence of acetylcholinesterase activity is shown in Fig. 2. Arrhenius plots exhibited a break point at 23.4°C for untreated synaptosomes and for synaptosomes treated with the cationic imipramine, but this shifted to around 16°C for synaptosomes treated with the other drugs. The biphasic nature of the Arrhenius plots for the acetylcholinesterase was abolished and a linear relation was obtained after solubilization of the enzyme with the non-anionic detergent Lubrol-PX, as described in the Materials and methods section. Table 1 shows that the

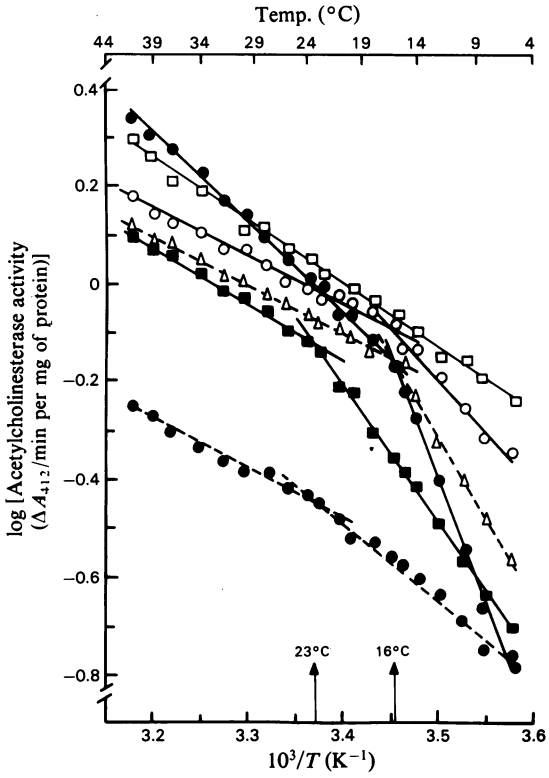

Fig. 2. *Effect of temperature on synaptosome-associated acetylcholinesterase activity in control (untreated) synaptosomes* (■——■), *in synaptosomes treated with phenobarbital* (●——●), *barbituric acid* (○——○), *primidone* (△---△) *or imipramine* (●---●), *and in synaptosomes solubilized by Lubrol-PX* (□——□)

For experimental details see the Materials and methods section. Each point represents the average value of duplicate determinations from a typical experiment which has been repeated three times. The straight lines were fitted by the method of least squares.

Arrhenius activation-energy values (E_a) in control (untreated) synaptosomes was 21.5±3.2kJ/mol above the break point and 49.7±7.3kJ/mol below the break point. In synaptosomes treated with 10mM-phenobarbital the E_a value was increased to 36.3±5.8kJ/mol above the break point and to 94.5±15.5kJ/mol below the break point. No statistically significant differences were shown in the E_a values of the other drugs tested above the break point, but below the break point the E_a for primidone (7mM)-treated synaptosomes was increased to 61.0±9.4kJ/mol, whereas for imipramine (10mM)-treated synaptosomes the E_a was decreased to 32.2±5.0kJ/mol. The above results show that the negatively charged drugs phenobarbital and barbituric acid as well as the neutral drug primidone caused a marked depression of the

Table 1. *Effect of charged drugs on the break point and activation energies derived from Arrhenius plots of the activity of brain synaptosome-associated acetylcholinesterase*

Values represent means ± S.D. for three independent experiments: *statistically significant compared with control ($P<0.05$).

Preparation	Break point (°C)	Activation energy (E_a) (kJ/mol)	
		Above break	Below break
Control (untreated synaptosomes)	23.4±0.6	21.5±3.2	49.7±7.3
+10mM-Phenobarbital	16.5±1.0	36.3±5.8*	94.5±15.5*
+10mM-Barbituric acid	16.3±1.2	18.5±1.0	41.0±7.0
+7mM-Primidone	16.3±1.5	19.0±1.5	61.0±9.4*
+10mM-Imipramine	23.0±1.7	19.8±2.1	32.2±5.0*

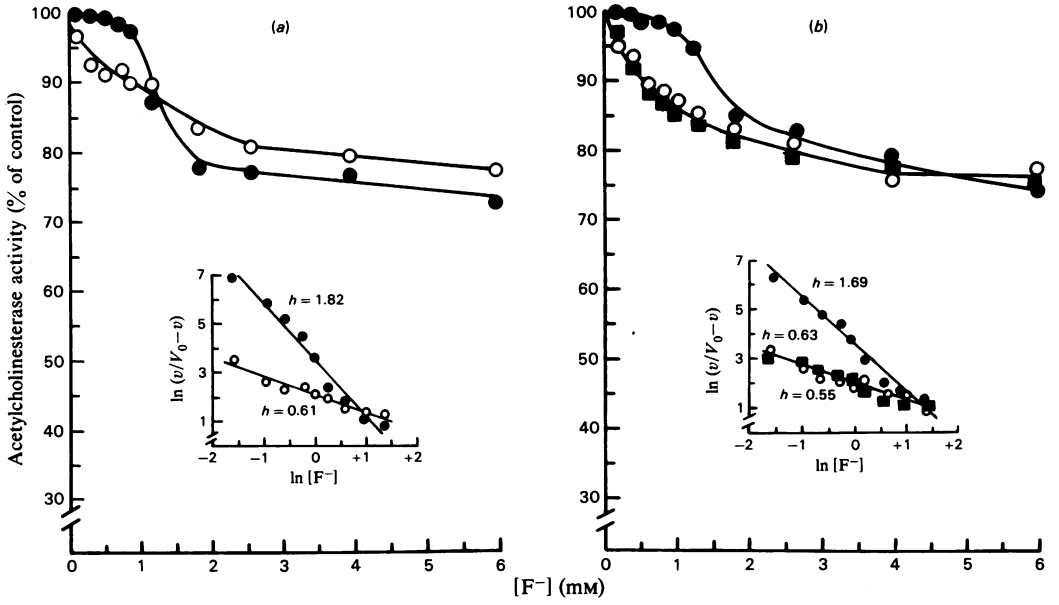

Fig. 3. *Effect of F^- on the reaction rate of the synaptosome-associated acetylcholinesterase activity (a) in control (untreated) synaptosomes (●) and in synaptosomes treated with phenobarbital (○), and (b) in synaptosomes treated with imipramine (●), primidone (■) or barbituric acid (○)*

The inserts show Hill plots of the same data. Corresponding Hill coefficients (h) are as indicated, The correlation coefficients (r^2) for the straight lines in the inserts are >0.95. v is the reaction velocity, and V_0 is the rate of the reaction in the absence of F^-. Points in the curves drawn are mean values of duplicate determinations from a typical experiment which has been repeated three times.

break point, but the positively charged imipramine gave a single well-defined break at about the same temperature found for the control synaptosomes (23.0°C). However, phenobarbital is an anionic compound that has been shown to increase the synaptosomal plasma-membrane fluidity (Deliconstantinos, 1983). Since discontinuities in Arrhenius plots of membrane-bound enzymes have widely been considered to reflect a lipid phase transition, and therefore to indicate a lipid-dependence of the enzymes (Shinitzky & Barenholz, 1978), the lowering of the break point produced by the negatively charged phenobarbital and barbituric acid and by the neutral primidone is consistent with asymmetric perturbations of the membrane lipid structure.

The co-operative behaviour of several membrane-bound enzymes could be used as a tool to detect modifications at the cell membrane level, since variations in Hill coefficients in co-operative membrane enzymes depend on their relationship to the lipid environment and on the fluidity condition of the latter (Farias, 1980; Deliconstantinos & Ramantanis, 1983; Tsakiris & Deliconstantinos, 1984). The allosteric inhibition of acetylcholinesterase activity by fluoride (F^-) was studied to detect a possible influence of the drugs on membrane fluidity. Fig. 3 shows the curves

obtained when the relative rates of the enzymic activity were plotted against different concentrations of F^- in control synaptosomes (Fig. 3*a*) and in those preincubated with the drugs for 1 h at 30°C (Figs. 3*a* and 3*b*). The Hill coefficient (h; slope) for the F^- inhibition of acetylcholinesterase activity for the control (untreated synaptosomes) was 1.82 ± 0.20, indicating the presence of co-operativity, which was abolished in the synaptosomes treated with the anionic drugs phenobarbital ($h = 0.61 \pm 0.09$) or barbituric acid ($h = 0.55 \pm 0.08$) and with the neutral primidone ($h = 0.63 \pm 0.10$). The effect of the cationic imipramine on the allosteric properties of acetylcholinesterase showed no statistically significant difference ($P > 0.05$) in Hill coefficient ($h = 1.69 \pm 0.16$) compared with the control synaptosomes.

These results may suggest that the anionic and neutral drugs increase the membrane fluidity and subsequently change the allosteric behaviour of the synaptosome-associated acetylcholinesterase, whereas the cationic drugs fail to influence the membrane fluidity.

Discussion

In synaptosomal plasma membranes the activity of acetylcholinesterase appears to be modulated by the physical properties of its lipid environment, as Arrhenius plots of the enzyme activity exhibit break points at temperatures corresponding to lipid-phase separations occurring in the membranes at 21.4°C (Pastuszko, 1980). Acetylcholinesterase is an integral enzyme with its active site exposed at the external cell surface, and it may be a biologically significant component of the membrane, contributing to its integrity and to the permeability changes occurring during transmission and conduction (Grafius *et al.*, 1971; Hollunger & Niklasson, 1973). Further support for the suggestion that brain acetylcholinesterase is an integral rather than a peripheral protein (Singer & Nicolson, 1972) comes from studies on the interaction of purified enzyme with liposomes of different composition, and from the effect of temperature on the activity of the reconstituted enzyme (Reavill *et al.*, 1978). It has been suggested that charged drugs may act preferentially at one or the other side of the plasma membrane, as the negatively charged (acidic) phospholipids predominate at the cytosol-facing surface (Rothman & Lenard, 1977). Consistent with such a suggestion is that the anionic phenobarbital can stimulate the activities of $(Na^+ + K^+)$-stimulated ATPase and Ca^{2+}-stimulated ATPase of synaptosomal plasma membranes (Deliconstantinos, 1983) and the activities of 5′-nucleotidase and glucagon-stimulated adenylate cyclase of liver plasma membranes (Dipple *et al.*, 1982; Houslay *et al.*, 1981). These effects appear to be mediated through changes in the membrane fluidity, and the stimulation of the enzyme activities that ensued was presumably due to an increase in the conformational flexibility of the enzyme achieved by a relief of a physical constraint imposed by the bilayer on the protein molecules.

On the basis of these considerations, in the present study the ability of the negatively charged drugs phenobarbital and barbituric acid, the neutral primidone and the positively charged imipramine to be associated *in vitro* with intact synaptosomes isolated from dog brain, causing functional changes of the synaptosome-associated acetylcholinesterase activity, was investigated. In addition, the evoked functional changes of the synaptosome-associated acetylcholinesterase by the positively charged phenothiazines perphenazine and trifluoperazine were also studied.

The curves representing the changes of acetylcholinesterase activity at different concentrations of the anionic drugs phenobarbital and barbituric acid and the neutral primidone have biphasic character (Fig. 1). Low concentrations of the drugs progressively activated the acetylcholinesterase activity; increasing the concentration above that maximally stimulating the enzyme activity caused a progressive inhibition with respect to the maximal stimulation. On the other hand, the positively charged drugs trifluoperazine and perphenazine gave a decrease of the enzyme activity, whereas no change was given by the positively charged imipramine. To investigate whether the effect of these drugs on acetylcholinesterase activity is consistent with an effect on the physicochemical characteristics of the membrane, and to discount the possibility that these drugs interact directly with the protein molecule to cause an irreversible change in its activity, the drugs were removed from the membrane after dilution or washing of the drug-loaded synaptosomes. The functional effect was fully reversible. Therefore the drugs do not irreversibly change the acetylcholinesterase activity, and their presence in the membrane causes a modulation of the enzyme activity. Moreover, solubilization of the acetylcholinesterase by using the non-ionic detergent Lubrol-PX resulted in no change in the enzyme activity at any charged drug concentration used. The results obtained for the effects of the charged drugs on the activity of the membrane-bound enzyme and on the solubilized enzyme may suggest that they exert their action not directly on the protein molecules but probably by changing the membrane lipid-bilayer microenvironment.

Abrupt changes in slope at a particular temperature have been taken to represent a phase transition in the lipid environment of membrane-

bound enzymes (Shinitzky & Barenholz, 1978). In the present study the acetylcholinesterase activity showed a break point at 23.4°C in untreated synaptosomes and in synaptosomes treated with the cationic imipramine, which was shifted to around 16°C in synaptosomes treated with the other drugs (Fig. 2). This would be expected from an increase in bilayer fluidity relieving a constraint on the protein, increasing its conformational flexibility and hence its activity (Dipple *et al.*, 1982; Deliconstantinos, 1983; Tsakiris & Deliconstantinos, 1984). Since acetylcholinesterase, which is a membrane enzyme with its active side exposed at the external leaflet of the bilayer, as mentioned above, is modulated by the negatively charged drugs phenobarbital and barbituric acid and by the neutral primidone, but not by the positively charged imipramine, we propose that the lipid-phase separation is localized to the external leaflet of the bilayer. This hypothesis is in line with the view of the known chemical asymmetry of the bilayer, in which negatively charged lipids predominate at the inner leaflet, whereas neutral and positively charged lipids are located in the external leaflet (Higgins & Evans, 1978). Since, however, the positively charged drugs trifluoperazine and perphenazine decrease the acetylcholinesterase activity, it is likely that the charged drugs are distributed between both halves of the bilayer of synaptosomal plasma membranes but exhibit a greater tendency to interact with one or the other half of the bilayer, thus causing functional modulation of the membrane enzymes and also changes in the lipid-phase separation. Moreover, it is well known that phenothiazines can selectively perturb lipid-bilayer membranes (Ahmed *et al.*, 1980), an effect that could cause the observed progressive inhibition of acetylcholinesterase activity. For this reason we decided to investigate further the effect of cationic drugs on the synaptosome-associated acetylcholinesterase activity by using the cationic imipramine.

In the present study, further evidence for the increase in the bilayer fluidity in synaptosomes treated with negatively charged drugs was obtained from the alterations in the co-operative behaviour of the synaptosome-associated enzyme acetylcholinesterase. As shown in Fig. 3, the value of the Hill coefficient, h, for the inhibition of acetylcholinesterase by F^- was decreased by the negatively charged drugs (phenobarbital, barbituric acid) and by the neutral primidone, indicating a loss of the co-operativity of the enzyme, consistent with a general increase in lipid fluidity. The lack of effect of the cationic imipramine on the allosteric properties of acetylcholinesterase suggests that this drug cannot influence the membrane fluidity.

Modulations of synaptosome-associated acetylcholinesterase activity caused by changes in lipid fluidity may influence the postsynaptic binding of acetylcholine to its receptors. Generally, modifications in membrane microenvironment by charged drugs could also alter the activities of other membrane-bound enzymes, binding of neurotransmitters, or conformation of receptors, which may account for the pharmacological effects of these charged drugs in the brain.

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