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Increase in acetylcholine concentrations in the brain of 'old' rats following treatment with pyrithioxin (Encephabol)

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1 Treatment of old rats, for two to three weeks, with pyrithioxin led to an increase in the levels of endogenous acetylcholine (ACh) in the cortex and the striatum but not in the hippocampus.

2 Pretreatment of old rats with pyrithioxin also increased the resting release and the K^+-stimulated release of radioactive ACh from brain slices *in vitro*.

Introduction

There is much evidence that disturbances in cerebral cholinergic neurotransmission are associated with senile dementia in humans. Also, in general no differentiation is now made between Alzheimer's disease and senile dementia and the term 'dementia of the Alzheimer type' (DAT) is often used. The relationship between damage to cholinergic pathways and DAT has been extensively reviewed (Marchbanks, 1982; Coyle *et al.*, 1983; Perry, 1986) and the most striking and consistent findings are marked deficits in both choline acetyltransferase (ChAT) and acetylcholinesterase in the cerebral cortex and in the hippocampus.

The compelling evidence for a cholinergic deficit in DAT does, of course, not rule out involvement of other neurotransmitter systems. Marchbanks (1982) discusses some of the work suggesting a catecholaminergic deficit and Rossor & Iversen (1984) review other non-cholinergic neurotransmitter abnormalities.

A related and important question is whether the degenerative processes associated with DAT are specific for this disease or whether they are merely an acceleration of the normal, progressive deterioration of mental processes that occur with aging. There is no clear answer to this question. However, in a recent critical review of the cholinergic hypothesis of geriatric memory dysfunction Bartus *et al.* (1982) came to the conclusion that there is considerable evidence suggesting that significant changes in cholinergic markers occur in the brains of aged animals and humans. In another recent study, involving mapping of neostriatal neurotransmitter systems as a function of aging, Strong *et al.* (1982) found a significant decrease in ChAT in the caudal striatum in rats aged 26 months compared with animals 6 months old. This study makes the very important point that age-related neurotransmitter changes in the neostriatum appear to be confined to discrete regions. One reason for the discrepancies in the results on neurochemical changes associated with aging, as well as with DAT, may well be indiscriminate pooling of heterogeneous subregions which exist within classically defined brain areas.

Another line of evidence linking the cholinergic system to learning and short term memory, functions readily lost during aging, comes from pharmacological studies. Cholinomimetic drugs are able to influence memory but the precise effects of the drugs are dependent on the 'age' of the memory, the drug dose, learning efficiency etc. and clear indications for any treatment have not yet been established (Squire & Davis, 1981; Iversen, 1986).

In view of the evidence linking the cholinergic system to certain age-related dysfunction we have examined the possibility that in 'old' rats cholinergic parameters might be affected by drug treatment. We concentrated initially on measurements of ACh content and ACh release since these parameters appeared most obviously relevant to cholinergic transmission. We investigated the possibility that treatment of old rats with pyrithioxin might have an effect on these parameters. Pyrithioxin has been used for a number of years as a CNS activating drug and there is some evidence that it has such an effect, especially in situations where CNS function is depressed, e.g. by anaesthetics (Martin, 1983).

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Methods

Treatment of animals

It was initially difficult to obtain a large number of old animals of the same strain. We therefore used for the first set of experiments, measuring ACh levels in the cortex only, male Wistar rats aged between 20 and 25 months; for all subsequent experiments Sprague-Dawley rats aged between 18 and 21 months were used. The animals were kept in separate cages and treated with pyrithioxin (daily dose 200 mg kg^{-1}) by mixing the drug with a small amount of cat food which was given as supplement to the normal CRM diet; the control animals received a similar amount of cat food. The animals took the drug completely and without any difficulties. All rats weighed between 500 and 700 g. After two to three weeks of treatment the animals were killed by decapitation.

Biochemical measurements

The brain was removed quickly, placed on an ice-cold stainless steel plate and immediately covered with ice-cold Krebs buffer containing 30 μM phospholine. Cerebral cortex, mainly frontal and parietal, as well as striatum and hippocampus were dissected out and the tissues, usually 30 to 50 mg, were homogenized in 400 μl of ice-cold Krebs buffer; half of the suspension was used for protein and, in some cases, ChAT determination while to the other half, 20 μl of trichloracetic acid (TCA, final concentration 5%) were added in preparation for ACh determination. ChAT activity was measured, at room temperature, according to Fonnum (1975) and protein according to Lowry *et al.* (1951).

Determination of acetylcholine

The method was basically as described by Israel & Lesbats (1982). After the addition of TCA the samples were left on ice for at least 1 h, centrifuged and the supernatants transferred to clean tubes. Then 10 μl of 0.5% sodium metaperiodate was added and the tubes again allowed to stand on ice for 30 min. The samples were then extracted with 2 ml water-saturated ether at least six times until the final pH was between 4 and 6. All traces of ether were evaporated from the aqueous phase with nitrogen. Aliquots, usually 15 μl, of the oxidised sample were processed exactly as described by Israel & Lesbats (1982). Light emission was measured using a Packard Picolite luminometer and each sample was compared with suitable standards.

Acetylcholine release from slices

The slices were prepared from about 500 mg of cerebral cortex using a McIlwain tissue chopper, cutting at 0.3 mm intervals and in two directions at a 45° angle. The slices were washed in cold buffer before being resuspended and incubated in 4 ml of oxygenated Krebs solution at 37°C for one hour; this buffer contained 2 μM [^{3}H]-choline. The slices were then washed several times with ice-cold, Ca^{2+} free, buffer (containing 100 μM physostigmine) to remove radioactivity that had not been taken up by the cells.

Following loading with [^{3}H]-choline the tissue was resuspended in 2 ml buffer and 200 μl aliquots were transferred to Eppendorf tubes; after centrifuging the tubes lightly the supernatant buffer was carefully removed and 0.2 ml of physostigmine containing 'release medium' – normal Krebs, high K^+ (35 mM) Krebs or a Ca^{2+} free buffer (containing 200 μM EGTA) – was added and release was started by incubating at 37°C. After 10 min the tubes were centrifuged in a Beckmann centrifuge and an aliquot of the supernatant was removed for further analysis; the pellets were treated with 5% TCA and allowed to stand in ice for about 60 min.

Extraction and separation of radioactive acetylcholine and choline

Radioactive ACh and choline were extracted following the procedures given by Marchbanks & Israel (1971) with minor modifications. The supernatants from the TCA-acidified samples were extracted with ether five times before ACh and choline were extracted with tetraphenylboron in allyl cyanide (20 mg ml^{-1}). Radioactive ACh and choline in the supernatants from the release experiments were extracted directly by shaking with tetraphenylboron in allyl cyanide. The radioactive compounds were re-extracted into aqueous solution with silver acetate. Following acidification with HCl the samples were washed several times with ether, freeze-dried and then dissolved in a small amount of methanol, containing carrier ACh and choline (12 mM). Aliquots were applied to thin layer chromatographic cellulose plates (E. Merck, Darmstad). The plates were eluted with *n*-butanol, glacial acetic acid, water, ethanol (8:1:3:2, v/v). The chromatograms were sprayed with an iodoplatinate reagent and the 'spots' eluted by leaving overnight in 1 ml of water. After adding 9 ml of Unisolve E (Koch-Light) the radioactivity was counted in the normal way. The R_F values found were: choline 0.31; ACh 0.43.

Internal standards were prepared, with every experiment, by adding known amounts of radioactive choline and ACh to blank samples before extraction. The percentage recovery was always about 60% for ACh and 70% for choline; the difference is the result of hydrolysis of ACh. Estimations for ACh and choline radioactivity in the samples were corrected accordingly.

Materials

Choline oxidase, horseradish peroxidase and luminol (5-amino 1, 2, 3, 4-terahydrophtalazindion-1, 4,) were obtained from Sigma. Acetylcholinesterase from Electrophorus electric organ (1000 u ml^{-1}) was from Boehringer; the solution (350 μl) was gel filtered on a 5 ml Sephadex G-50 coarse column and collected in 1 ml. ACh was from Sigma, other chemicals were AR grade and obtained from Fisons. [^{3}H]-choline (75–85 Ci $mmol^{-1}$) and [^{3}H]-ACh (70–80 Ci $mmol^{-1}$) were obtained from the Radiochemical Centre, Amersham. Phospholine (ecothiopate iodide) was from Ayerst Laboratories, Andover, Hants. Phyrithioxin was a gift from E. Merck, Darmstadt.

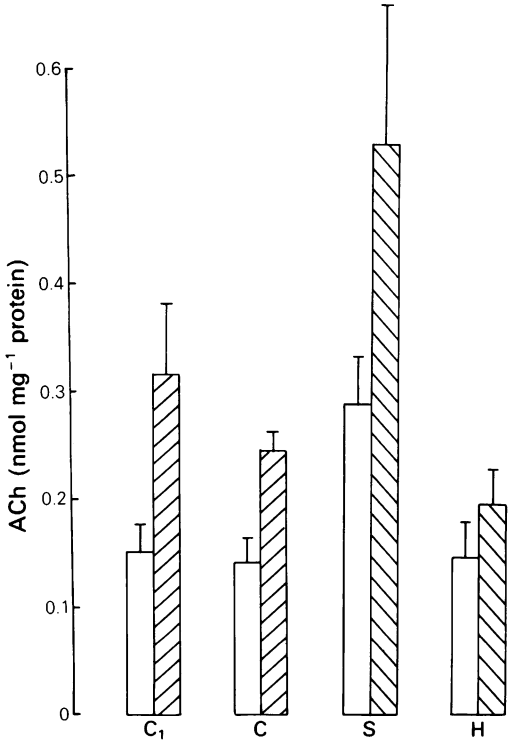

Figure 1 The effects of pyrithioxin treatment (200 mg kg^{-1} per day for two weeks) on acetylcholine (ACh) concentrations in the cortex of old Wistar rats (C_1) and cortex (C), striatum (S) and hippocampus (H) of old Sprague-Dawley rats. Each column represents the mean with s.e.mean indicated by the vertical lines. Eight or nine animals were used to obtain each result. In the cortex and striatum the increases resulting from treatment (hatched columns are significant at $P<0.05$ compared to untreated animals (open columns).

Results

Figure 1 shows the effects of treating old rats with pyrithioxin on ACh levels in various areas of the brain. The treatment significantly increased ACh concentrations in the cortex and in the striatum but not in the hippocampus.

We measured ChAT activity in the cortex, striatum and hippocampus of eight control and eight treated animals to see whether the observed increase in ACh levels was associated with an increase in the activity of this ACh synthesizing enzyme. We found no significant difference between the two groups (Table 1).

ACh in the synaptic terminal exists in different compartments and an increase in total ACh content does not necessarily indicate an increase in the amount of ACh available for release. We. therefore, investigated the effects of pyrithioxin-treatment on the release of ACh. Slices obtained from the cortex of treated and untreated animals we incubated with [^{3}H]-choline for one hour before resting and K^+ stimulated release of [^{3}H]-ACh were measured. The results are given in Table 2. Both resting and K^+ stimulated release were higher in the tissue obtained from the treated animals; there was no difference when release was measured in the absence of calcium. The uptake of [^{3}H]-choline and its conversion into ACh were similar in cortical slices from treated and untreated animals; it was also assumed that during the one hour incubation in the [^{3}H]-choline-containing buffer all intracellular ACh would have been labelled (Weiler *et al.*, 1983). However, we have no information on the specific activity of either the [^{3}H]-choline in the pool from which it is taken for acetylation or on the [^{3}H]-ACh released. The data of the release experiments shown in Table 2 therefore indicate that treatment with pyrithioxin affects mechanisms involved in Ca^{2+} dependent ACh release, but it was not possible to calculate the actual amounts of ACh released.

Table 1 The effects of pyrithioxin treatment (200 mg kg^{-1} per day for two weeks) on choline acetyltransferase (ChAT) activity

	ChAT activity (nmol mg^{-1} protein h^{-1})	
	Control	*Treated*
Cortex	7.5 ± 0.63	9.8 ± 0.71
Striatum	16.3 ± 1.84	19.4 ± 1.95
Hippocampus	6.7 ± 0.71	8.1 ± 0.75

Eight animals were used to obtain each result presented.

Table 2 The release of labelled choline and acetylcholine (ACh) from cortical slices obtained from untreated and treated (pyrithioxin, 200 mg kg^{-1} per day, for two to three weeks) old rats

	Release of [^{3}H]-ACh and [^{3}H]-choline			
	Control animals		*Treated animals*	
	Fractional ACh release	*Fractional choline release*	*Fractional ACh release*	*Fractional choline release*
Resting	0.079 ± 0.015	0.436 ± 0.039	0.115 ± 0.019*	0.441 ± 0.048
High K^+	0.248 ± 0.026	0.408 ± 0.025	0.386 ± 0.40*	0.453 ± 0.039
High K^+ and no Ca^{2+}	0.095 ± 0.025	0.380 ± 0.082	0.128 ± 0.041	0.453 ± 0.066
	Conversion [^{3}H]-choline to [^{3}H]-ACh: 0.741 ± 0.017		Conversion [^{3}H]-choline to [^{3}H]-ACh: 0.805 ± 0.019	

Release was measured over a 10 min period and is expressed as fractional release (radioactivity released as a fraction of the radioactivity present in the tissue at the beginning of the release period). The measurements were done in triplicate and ten or eleven animals were used to obtain each result presented. The differences in ACh release between tissues obtained from treated and untreated animals are significant (*$P < 0.01$) for both resting release and K^+ stimulatd release in the presence of Ca^{2+}. The amount of radioactivity taken up during the preceding 60 min loading period was not significantly different for the two types of tissue.

Discussion

The results presented here suggest that the administration of pyrithioxin, over a period of two weeks, leads to an increase in ACh levels in the cortex and in the striatum, and that following treatment the *in vitro* release of [^{3}H]-ACh from cortical slices is facilitated. The mechanisms by which the drug might produce these effects are not known. Changes in cerebral ACh levels have been observed following the administration of drugs that act primarily on ACh release. However, the general pattern seems to be that any procedure that will facilitate transmission at cholinergic synapses in the CNS, as indicated by an increase in ACh release and ACh turnover, will also produce a decrease in ACh levels in the relevant region. This has been observed when dopamine antagonists were used to reduce the tonic inhibitory influence that is exerted by the dopaminergic nigro-striatal pathway on cholinergic neurones in the striatum (Scatton, 1982), and when γ-aminobutyric acid (GABA)-mimetic agents acted directly on cholinergic synapses in the striatum (Scatton & Bartholini, 1980). The reverse has also been found: activation of the dopaminergic pathways referred to has been shown to reduce both release and turnover of ACh and at the same time increase ACh concentrations (Scatton, 1982). The pattern is probably similar when anaesthetics reduce ACh release and turnover, not by activating or blocking a particular and specific set of receptors but by interfering in a more non-specific way with synaptic transmission (Schmidt, 1966). The cholinergic synapses in the CNS apparently differ in this respect from those at the neuromuscular junction and in ganglia where the ACh content of the nerve ending is kept fairly constant over a wide range of nerve activity (Tucek, 1984).

The decrease in ACh concentration in the nerve ending following increased release is believed to be the result of synthesis not keeping up with the increased demand. Support for this interpretation comes from the observation that drugs that reduce ACh levels in certain brain regions by increasing release have less effect on ACh concentrations if the animals are pretreated with choline (for references see Tucek, 1984). In most studies establishing the described relationship between ACh levels and release in the CNS, ACh levels were measured shortly after the release had been altered.

One of the few investigations concerned with the temporal dependance of effects on ACh levels describes the time-course of the prophylactic effect of choline administrationin attenuating the atropine-induced depletion of ACh (Schmidt & Wecker, 1981). The data show that the effects of choline administration on cerebral ACh metabolism are not mediated directly through increased total free brain choline but lend strong support to the suggestion that there is a phospholipid pool from which choline can be made available for ACh synthesis. The existence of such a phospholipid pool may help to explain how a drug can, in the long term, facilitate ACh release and increase ACh concentrations. If a drug does affect the metabolism of membrane phospholipids it is possible that this may have consequences both for release mechanisms and the availability of choline. There is evidence that chronic administration of pyrithioxin has an effect on membrane phospholipids; Woelk (1984) found that, in old rats, the incorporation of

intracisternally administered ^{32}P into neuronal phosphatidylcholine and phosphatidylserine was significantly increased by pretreatment of the animals with pyrithioxin.

The interpretation of data on the *in vitro* release of [^{3}H]-ACh following incubation of slices with [^{3}H]-choline is complicated by uncertainties about the pool size of endogenous choline and ACh. We, therefore, began to measure the release of endogenous ACh using the chemiluminescent method. Interpretation of these experiments was, however, less straight forward than might be anticipated. Apart from the need to correct for substantial amounts of choline we found that *post mortem* changes in the level of endogenous ACh were very rapid so that by the time release was measured intracellular ACh levels were very different from what they were immediately after killing the animal. Any pre-incubation assuring reproducible release patterns led to a substantial increase in intracellular ACh even when no exogenous choline was present; this is in agreement with the observations of Weiler *et al.* (1979). It is known that ACh concentrations in the brain change very rapidly after death and the importance of the method of killing has frequently been emphasized. ACh concentrations found after decapitation are always considerably lower than those found after microwave irradiation but the effects of drugs appear to be very similar regardless of the method of killing (Glick *et al.* 1976; Scatton & Bartholini, 1980). The ACh concentrations presented in this paper are very similar to those generally found after decapitation.

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