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UPTAKE OF 5-HYDROXYTRYPTAMINE IN THE CATECHOLAMINE CONTAINING AREAS OF THE HYPOTHALAMUS OF THE RAT AFTER TREATMENT WITH PHENELZINE AND TRYPTOPHAN

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1 Uptake of 5-hydroxytryptamine in the catecholamine containing nerve endings of the hypothalamus in the rat brain was found after intraperitoneal injection of phenelzine sulphate (25 mg/kg) and tryptophan (100 and 400 mg/kg).

2 The results were obtained by fluorescence microscopy and microspectrofluorimetry.

Introduction

As our knowledge of the pharmacology of catecholamine (CA) systems in the brain has grown, so the 'Amine Theory of Affective Disorders' has been subjected to more detailed development. The efficacy of a combination of a monoamine oxidase inhibitor (MAOI) and tryptophan loading in depression was demonstrated by Coppen, Shaw & Farrell (1963), by Glassman & Platman (1969) and by Murphy, Baker, Kotin & Bunney (1973). It has been suggested that the increase in brain 5-hydroxytryptamine (5-HT) which this treatment causes in animals (Ashcroft, Eccleston & Crawford, 1965) might be the therapeutic agent. In a recent paper, Eccleston & Nicolaou (1978) showed that although 5-HT levels do rise after such treatment, the rise is associated with an increase of concentrations of CA metabolites, suggesting that the release of CA is another possible therapeutic effect. Such a mechanism was actually proposed by one of the earliest clinical investigations of the efficacy of combined tryptophan and MAOI (Pare, 1963).

There have been several reports that 5-HT can be accumulated within the terminals of CA neurones, both *in vivo* and *in vitro* (Bertler, Falck & Owman, 1964; Fuxe, Butcher & Engel, 1971; Butcher, Engel & Fuxe, 1972; Dow & Laszlo, 1976) and we undertook these experiments with the intention of showing that this phenomenon occurred after a MAOI and tryptophan treatment and that it could be studied histochemically within small parts of sections of brain by a method of microspectrofluorimetric analysis already described (Laszlo & Arbuthnott, 1980).

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Our demonstration of the simultaneous presence of 5-HT and CA in terminal regions adds to the complexity of the interpretation of experiments which employ tryptophan loading as a means of studying the behavioural results of 5-HT release. As well as the neuroanatomical evidence for 5-HT/dopamine interactions (see Nicolaou, Garcia-Munoz, Arbuthnott & Eccleston, 1979), it seems that a purely pharmacological interaction occurs and that 5-HT is formed not only in the 5-HT containing area of brain after tryptophan loading. If its formulation in CA terminals is associated with the release of catecholamines (Eccleston & Nicolaou, 1978) then the blockade by neuroleptic drugs of the behavioural syndrome which follows administration of a MAOI and tryptophan (for review see Green, 1978) may reflect physiological interactions between the two systems poorly, if at all.

Methods

Animal pretreatment

Albino Wistar male rats (21) weighing between 215 and 320 g were used. Control tissue from untreated rats was routinely processed along with the brains from experimental rats.

In preliminary work, the emission spectrum from 5-HT containing cells of the raphe nuclei in rat brain was recorded in animals pretreated with nialamide (Niamid, Pfizer, 500 mg/kg i.p. dissolved in saline) 5 h 20 min before death.

The experimental animals were injected (i.p.) with 25 mg/kg phenelzine sulphate 1 h before tryptophan (100 or 400 mg/kg) and killed 1 h later by decapitation

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under ether anaesthesia. The brains were dissected and small areas of brain prepared for fluorescence histochemistry according to Laszlo (1974). The areas of brain were freeze-dried and exposed to formaldehyde vapour. Sections 8 μm thick were cut from the tissue after paraffin embedding *in vacuo*.

Fluorescence microscopy

Sections were studied by transmitted illumination in a Large Zeiss Fluorescence Microscope. Fluorescence was produced by an (Osram HBO) 200 W mercury lamp with a BG3 (Schott) primary and 50 (Zeiss) secondary filters and observed with a ×10 FL-Neofluar (n.a. 0.30) and a ×40 immersion objective (n.a. 1.0) with ×8 eye piece, with bright field condenser (n.a. 1.4) and also with a cardioid immersion condenser (n.a. 1.2/1.4).

The areas to be studied were identified by staining adjacent sections by a shortened version of the toluidine blue staining described earlier (Laszlo, 1977). The sections were treated with (1) xylene; (2) ethanol (absolute); (3) ethanol (50%); (4) water; (5) toluidine blue (0.2%); (6) ethanol (50%); (7) ethanol (absolute); (8) xylene. Sections were kept for 10 to 20 s in each solution, except in toluidine blue, in which they were kept for 30 s; then they were mounted in Canada balsam.

Microspectrofluorimetry

Fluorescence spectra were recorded and intensity of fluorescence was measured at specific wavelengths from monoamine containing structures of the brain and from standard droplets containing noradrenaline (NA; noradrenaline bitartate 1.0 mg/ml) and 5-HT (5-hydroxytryptamine creatinine sulphate 1.0 mg/ml) in 2% albumin. An Osram HBO 100 w/a mercury lamp produced the fluorescence and a dark field cardioid immersion condenser (n.a. 1.2/1.4) a ×40 immersion objective (n.a. 1.0) were used with a UG1 (Schott) primary and a 41 (Zeiss) secondary filter; fluorescence spectra were recorded between 400 and 650 nm, at 5 to 10 nm intervals with a continuous interference filter as described by Laszlo (1974). A 0.16 mm diameter diaphragm was used in the photometer head which corresponded to 3.2 μm diameter area in the section from which the intensity of fluorescence was measured by the modified microspectrophotofluorimeter described by Laszlo & Arbuthnott (1980).

Results

Control

Fluorescence microscopy: sections from untreated (control) rats showed in the anterior hypothalamus the specific green fluorescent nerve endings around non-fluorescent cells of the nucleus supraopticus and fine green yellow fluorescent nerve endings in the wall of the third ventricle as described by Carlsson, Falck & Hillarp (1962).

Microspectrofluorimetry

Fluorescence spectra were recorded from droplets containing NA and 5-HT and from the fluorescent structure containing monoamine in tissue sections. The maximum of the fluorescence spectra of NA in droplets was found between 470 and 475 nm, while for 5-HT it was between 510 and 520 nm. NA nerve endings around the artery at the ventral surface of the brain showed a similar spectra to those of NA standards, although the spectra from the tissue sections showed a slight shift towards the shorter wavelegths of approximately 10 nm.

Nerve endings in the nucleus supraopticus of the anterior hypothalamus had fluorescence spectra with a maximum between 460 and 470 nm. These spectra, when compared to those of the standards showed a shift of approximately 10 to 15 nm toward the shorter wavelengths. Spectra recorded from sections compared to those of the corresponding standard monoamines were shifted due to the non-specific fluorescence in the sections (Laszlo, 1974). In these experiments, the spectra were recorded immediately after mounting the sections. Spectra recorded one day later showed a further shift by approximately 10 nm toward the shorter wavelength (maximum between 450 and 460 nm). The CA fluorophore in the mounted sections is known to decompose faster than the non-specific fluorescence which was found to be more stable (Falck & Owman, 1965).

Fluorescence spectra of yellow fluorescent 5-HT cells of the raphe nucleus from rats treated with nialamide were also recorded. The spectra of the 5-HT cells had a wider maxima than those of the NA terminals and they also showed a considerable variation. In agreement with Björklund, Flack & Stenevi (1971) the peak fluorescence was about 510 nm in these cells corresponding to that of the 5-HT containing standard droplets.

The 5-HT content of a fluorescent structure in the presence of catecholamines can be described by the relative intensity of fluorescence at 530 nm ($F_{\lambda 530}/F_{\lambda max}$ the ratio of the intensity of fluorescence at one specified wavelength to that of the maximum of fluorescence), described by Laszlo & Arbuthnott (1980). This was calculated from the recorded spectra of 5-HT containing cells of the raphe nuclei and was found to be 0.770 ± 0.083 standard deviation (s.d.), the number of measurements (n) was 15.

Treatment with phenelzine

Injection of phenelzine sulphate (25 mg/kg) did not appear to cause any change of monoamine content in the areas studied, since there was no difference either in the fluorescence picture or in the spectra recorded in tissue from treated rats compared to those of the controls.

Treatment with phenelzine (25 mg/kg) and tryptophan (100 mg/kg)

The anterior hypothalamus of the treated rats contained yellow green fluorescent granules and increased number of green fluorescent granules of the nerve endings in the nucleus supraopticus. The control sections in the corresponding area contained green fluorescent nerve endings in lesser numbers and areas corresponding to these nerve endings showed only diffuse green fluorescence. The background fluorescence of the control rat was less intense than that of the treated one. There was no detectable change in the spectra recorded.

Treatment with phenelzine (25 mg/kg) and tryptophan (400 mg/kg)

The whole section from the treated rat was found to be brighter than from the control. Fine greenish yellow fluorescent nerve endings were present in the nucleus supraopticus of the treated rat which exhibited more intense fluorescence than those from the control animal. After treatment, the nucleus supraopticus exhibited more intense yellowish fluorescence than the control.

Spectra were recorded from the ventral area under the third ventricle. Preliminary comparison of the spectra recorded from fluorescent structure of the treated and control rat showed a partial shift of the spectra from the treated rat towards the longer wavelength. This change of spectra was calculated by measuring the ratio of the relative intensity of fluorescence at one wavelength, to that of the maximum ($F_{\lambda 535}/F_{\lambda max}$) from the treated and from the control rat. The intensity of fluorescence was measured in the range where the relative intensity of fluorescence between NA and 5-HT is the greatest (Laszlo & Arbuthnott, 1980). The mean of the values of ($F_{\lambda 535}/F_{\lambda max}$) in this experiment in the treated rat was found to be higher than in the control (Table 1, experiment 1).

The previous experiment was repeated, but on this occasion the fluorescence intensity was measured only at one wavelength according to the procedure described by Laszlo & Arbuthnott (1980). The fluorescence of the sections from treated rats showed a change similar to that of the control, as was found in the first experiment. In the treated rat, the area under the third ventricle showed a green yellow fluorescent layer while it showed green fluorescence in the sections from the control rat.

The mean of ($F_{\lambda 530}/F_{\lambda max}$) values of the treated rats was measured and compared to that of the controls. Any measurements taken from adjacent areas were eliminated from the calculation of the results, since they could have influenced the subsequent readings due to the effect of u.v. light on the fluorophore. The difference between the two series of measurements (Table 1, experiment 2) was found to be highly significant. The increase of the relative intensity of

Table 1 Effect of intraperitoneal injection of phenelzine sulphate (25 mg/kg) and tryptophan (400 mg/kg) on the relative intensity of fluorescence in nerve endings at specified wavelengths.

Area	*Bottom of the 3rd ventricle*		*Lateral area of external layer of the median eminence*	*Supraoptic nucleus*
Experiment	1 §	2 **	3 **	4 **
Formula	$\frac{F_{\lambda 535}}{F_{\lambda max}}$ *	$\frac{F_{\lambda 530}}{F_{\lambda max}}$ *	$\frac{F_{\lambda 535}}{F_{\lambda max}}$ *	$\frac{F_{\lambda 535}}{F_{\lambda max}}$ *
$\bar{x}_t$ †	0.3308 ± 0.0384 (12)	0.395 ± 0.044 (16)	0.3718 ± 0.0672 (33)	0.2591 ± 0.0381 (23)
$\bar{x}_c$ ‡	0.2400 ± 0.0630 (11)	0.327 ± 0.072 (16)	0.3254 ± 0.0525 (30)	0.185 ± 0.0904 (12)
t test (2 tailed) *P*	< 0.001	< 0.005	< 0.005	< 0.005

* Intensity of fluorescence at 535 or at 530 nm in relation to that at the maximum.
† Mean of the fluorescence intensity in sections from treated animals.
‡ Mean of the fluorescence intensity in sections from control ± s.d. (number of measurements).
§ Intensity of fluorescence measured from the whole spectra.
** Fluorescence intensity measured only at the specified wavelengths.

fluorescence in the treated animals indicates the shift of the fluorescence spectra towards the longer wavelength due to the uptake of 5-HT.

In experiments 1 and 2 (Table 1) the fluorescence intensity was measured from an area ventral to the third ventricle, selected without regard to the organisation of the structure in a medio-lateral plane. Experiment 3 (Table 1) demonstrated that the shift in the fluorescence spectra was also visible in the lateral area of the external layer alone.

Changes in the fluorescence spectra were also seen in the supraoptic nucleus, an area thought to contain only NA. The results in this case (Table 1, experiment 4) also suggest the uptake of 5-HT.

Total monoamine concentration was estimated from the intensity of the fluorescence calculated from the amplification necessary to record the spectra. A comparison of F_{max} of the treated to control animals shows a 2.4 fold increase in fluorescence in the median eminence and approximately 2 fold in the supraoptic nucleus.

Discussion

The localization of the uptake of 5-HT at cellular level was studied after the treatment of rats with tryptophan, the precursor of 5-HT and phenelzine, a MAOI, which prevents the enzymatic breakdown of this monoamine. In similar experiments Hess & Doepfner (1961), Ashcroft *et al.* (1965) and Collard & Roberts (1974) measured the accumulation of 5-HT in the brain by chemical estimation. The increase of 5-HT in such experiments in the 5-HT containing neurone system was described by Aghajanian & Asher (1971), Aghajanian, Kuhar & Roth (1973) and also after treatment with 5-hydroxytryptophan (the precursor of 5-HT) an increase of fluorescence in various structures in rat brain was shown by Fuxe (1965). Due to non-specific uptake, 5-HT can also be found in areas normally containing only catecholamines (Bertler *et al.*, 1964; Fuxe *et al.*, 1971; Butcher *et al.*, 1972; Dow & Laszlo, 1976). The site of this non-specific uptake was studied in this work in which the uptake was evaluated by measurement of the relative intensity of fluorescence at one wavelength as described by Laszlo & Arbuthnott (1980). This method makes the measurements and the characterization of the fluorescence quicker and also more accurate than recording of the whole spectra, especially in the case of 5-HT containing structures since fluorescence due to this monoamine fades much faster than that due to CA. Quantifying 5-HT in the presence of NA in model droplets and in tissue sections recently has been described by Geyer, Dawsey & Mandell (1978) by measuring their fading. The method is based on the difference between the fading of fluorescence of the two amines and requires 14 s exposure to u.v. light. Our procedure is more rapid since it requires only three discrete measures of fluorescence (baseline, 530 nm and maximum).

An increase in fluorescence intensity was visible after 25 mg/kg phenelzine and 100 mg/kg tryptophan but this was not sufficient to be detectable as a change in the fluorescence spectra recorded. However, the time required to process the tissue sections and to position the fluorescent structure in the measuring field is considerably greater than for droplets, and it may have caused the 5-HT fluorophore to fade below the detectable limit (25% 5-HT in droplets, see Laszlo & Arbuthnott, 1980). Without concomitant biochemical determinations, we cannot be sure that the 5-HT content has reached even 25% of the amine in terminals in these animals.

The larger dose of tryptophan (400 mg/kg) did lead to a detectable change in the spectra of the emitted fluorescence, in phenelzine-treated rats. Such a change is associated with the addition of 5-HT to model droplets containing CA. In tissue this contamination is most likely to have been caused by the uptake of 5-HT into CA containing nerve terminals. A very large increase in the 5-HT content of the extracellular space might cause sufficient change in the spectra but in view of the concentration of dopamine in terminals and of our 25% detection limit, this seems unlikely. Another possibility is that 5-HT was taken up into 5-HT containing terminals which were normally below the detection limit for the methods. This too seems unlikely because 5-HT was not detected in these areas even after MAOI treatment.

Between experiments 2 and 3 we became aware of the work of Sladek, Sladek, McNeill & Wood (1978) who provide evidence for an accumulation of 5-HT in tanycytes of the median eminence. The ventrolateral area chosen in experiment 3 is some way from the area in which 5-HT accumulation is described in their paper but the shift in the spectrum of the emitted fluorescence is still present. It is likewise detectable in the supraoptic nucleus which is rich in NA containing terminals (Fuxe, 1965; Carlsson *et al.*, 1962), even when the measurements are made in an area well clear of the 5-HT containing suprachiasmatic nucleus.

In these experiments, in contrast to previous work (Fuxe & Ungerstedt, 1968), the animals were treated only with phenelzine and tryptophan. The measurement of spectral shift made pretreatment with reserpine unnecessary. Although the total uptake of 5-HT may have been reduced compared with earlier work it still occurs even in the absence of reserpine and with intact CA stores.

If the 5-HT is within the terminals then it could be the cause of release of CA (Eccleston & Nicolaou, 1978) by simple displacement. However, the biochemical experiments detected no loss of CA, only an increase of metabolite concentration so that some

compensatory mechanism must be at work. It is not at all clear whether the 5-HT which we detect is first synthesized at some other site and then taken up or whether the synthesis occurs via the aromatic-acid decarboxylase which would normally act on L-DOPA within CA neurones. At least some part of the synthesis is likely to have been accomplished outside the CA nerve endings since tyrosine hydroxylase has a low affinity for tryptophan. By whatever mechanism it occurs, the presence of 5-HT in nerve terminals which usually contain CA and the concomitant increased release of CA makes the interpretation of precursor loading experiments extremely difficult.

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