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THE USE OF STEREOTACTIC DISSECTION FOLLOWED BY FLUORIMETRIC ASSAY, TO DETERMINE THE DISTRIBUTION OF NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE IN THE PREOPTIC HYPOTHALAMIC AREA OF RABBIT BRAIN: AN ALTERNATIVE APPROACH TO HISTOCHEMISTRY

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1 A method of stereotactic dissection followed by fluorimetric assay, has been used to determine the distribution of noradrenaline, dopamine and 5-hydroxytryptamine (5-HT) within the preoptic hypothalamic area of rabbit brain.

2 The method involved subdividing the area into a number of thin slices cut with reference to stereotactic co-ordinates. The amine content of each slice was then determined fluorimetrically.

3 Noradrenaline was shown to be concentrated (>2.0 μg/g) in two areas within the preoptic hypothalamic area, both of which were adjacent to the mid-saggittal line: the first was close to the preoptic nucleus and the second immediately caudal to the main group of hypothalamic nuclei.

4 Dopamine was evenly distributed throughout the preoptic hypothalamic area.

5 5-HT was concentrated (>1.6 μg/g) towards the lateral borders of the area, principally in the region which joins the preoptic area to the anterior hypothalamus.

6 The relative merits of the stereotactic approach and the histochemical approach are discussed.

Introduction

The concentration of noradrenaline, dopamine and 5-hydroxytryptamine (5-HT) in different parts of the mammalian brain was reported by Vogt (1954), Carlsson (1959) and Amin, Crawford & Gaddum (1954) respectively, and their results have subsequently been confirmed by other investigators using a variety of species. It was found that all three amines were unevenly distributed throughout the brain and that the highest concentration of both noradrenaline and 5-HT occurred in the hypothalamus. Dopamine, although present in the hypothalamus, was located predominantly in the caudate nucleus. Anatomically, the hypothalamus is a complex region associated with many physiological and psychological functions. Brodie & Shore (1957) have suggested that noradrenaline and 5-HT function as hypothalamic transmitters. In order to examine this possibility it is desirable to establish the precise location of these amines within this area of the brain. One approach to this problem was the development of the fluorescence histochemical technique (Falck, 1962) for the location of catecholamines and 5-HT in neurones. Using this technique, Andén, Dahlström, Fuxe, Larsson, Olson & Ungerstedt (1966) prepared a map of the neuronal systems in the CNS containing the three amines, and Fuxe, Hökfelt & Ungerstedt (1968) described the intra-hypothalamic distribution of these amines in some detail. An alternative approach to histochemistry was suggested by the report of a stereotactic method of dissection for brain tissue (Ridge, 1964). This method involved subdividing a brain region into a number of thin slices cut with reference to stereotactic co-ordinates. This paper describes results obtained when the distribution of noradrenaline, dopamine and 5-HT within the preoptic hypothalamic region of rabbit brain was investigated by the method of stereotactic dissection followed by fluorimetric assay.

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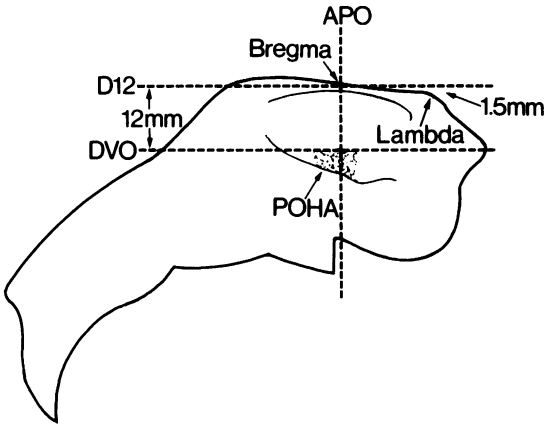

Fig. 1 Stereotactic reference planes for the rabbit head as defined by Sawyer *et al.* (1954). The head is levelled so that the bared skull at Bregma is 1.5 mm higher than the bared skull at Lambda. In this position the frontal zero plane (APO) passes through the plane of the coronal suture, the sagittal zero plane (MSO i.e. the plane of the paper) passes through the plane of the mid-sagittal suture and the horizontal zero plane (DVO) lies in an arbitarily chosen plane 12 mm below the surface of the skull at Bregma. POHA = preoptic hypothalamic area.

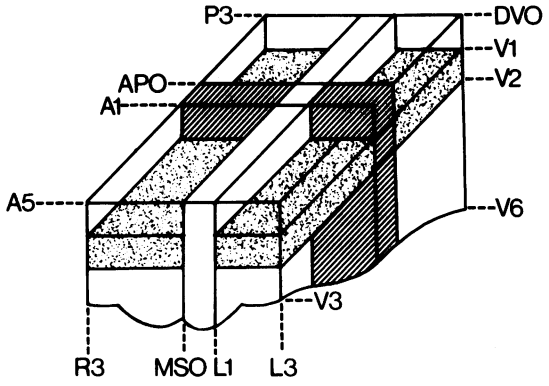

Fig. 2 The stereotactic co-ordinates of the preoptic hypothalamic area. Co-ordinates are expressed in mm. MSO, DVO and APO are the mid-sagittal, dorsal-ventral and anterior-posterior reference planes referred to in Figure 1. The preoptic hypothalamic area is contained between the frontal co-ordinates A5 and P3, sagittal co-ordinates L3 and R3, horizontal co-ordinates DVO and the base of the brain. The cross hatched area represents a 1 mm frontal slice cut between A1 and APO, the stippled area represents a 1 mm horizontal slice cut between V1 and V2, and the unshaded area represents a 1 mm sagittal slice cut between MSO and L1.

Methods

Materials

In order to minimize anatomical variation between animals, only healthy female New Zealand White rabbits between 3.0-3.6 kg were used. They were killed by an intravenous injection of air.

Stereotactic co-ordinates

Sawyer, Everett & Green (1954) defined three mutually orthogonal reference planes for stereotactic studies on the rabbit diencephalon (Figure 1). These reference planes were used again by Ridge (1964) to prepare his atlas of whole rabbit brain and have been retained in the present study. The limits of the preoptic hypothalamic area (POHA) to be considered in this study were defined as A5 to P3, L3 to R3 and DVO to the base of the brain (Figure 2). A5 indicates a plane parallel and 5 mm anterior to the frontal zero plane, and P3 indicates a plane parallel and 3 mm posterior to the frontal zero plane. Similarly, L3 and R3 indicate planes parallel and 3 mm to the left or right of the mid-sagittal zero plane. DVO is the dorso-ventral zero plane.

Stereotactic dissection

Blocks of tissue representing the POHA and defined by the co-ordinates A5, P3, L3, R3, DVO and the base of the brain were dissected out and subdivided into slices as described by Ridge (1964). In one series of experiments the POHA was divided into eight frontal slices; in a second series into six sagittal slices; and in a third series into six horizontal slices. All slices were 1 mm thick except the most ventral horizontal slice which was the irregular shaped piece of tissue between V5 and the base of the brain. The cross hatched area in Fig. 2 indicates the volume occupied by a 1 mm frontal slice cut between the co-ordinates A1 and APO. Such a sample slice would contain all the anatomical features contained between A1 and APO, L3 and R3, DVO and the base of the brain. Similarly, a sagittal slice cut between the MSO and L1 will contain all the anatomical features contained in a volume represented by the unshaded portion; and a horizontal slice cut between V2 and V1 will contain the anatomical features found within the volume represented by the stippled area. Each slice was assayed separately for noradrenaline, dopamine and 5-HT.

Extraction and assay

Noradrenaline, dopamine and 5-HT were estimated concurrently as described by Metcalf (1974).

Results

Stereotactic dissection technique

The reproducibility of the method for subdividing the POHA is shown in Table 1, which compares the total weight of the POHA when dissected as frontal, sagittal or horizontal slices with the weight of the area when dissected as a single sample. In no case was there a significant difference ($P>0.05$) between the weight of an area obtained as the sum of the individual slices and that of the area dissected as a whole.

Validity of the technique

A test of the validity of subdividing an anatomically complex area and determining the amine content of the very small tissue samples produced is given in Table 2. The amine content of the POHA is the same whether it is dissected as a single sample or computed by adding together the amount of amine found in the individual slices. The results presented in this table also confirm that the amine assays are valid at the low concentrations present in the tissue slices.

The distribution of amines within the preoptic hypothalamic area

The concentrations of noradrenaline, dopamine and 5-HT found in frontal, sagittal and horizontal slices cut from the POHA are shown in Figure 3.

Frontal slices (Figure 3a)

5-HT is evenly distributed throughout the POHA in a rostro-caudal direction. In contrast, noradrenaline appears to be bi-modally distributed with an area of high concentration between A4 and A2 and another around APO-P1. The concentration of noradrenaline is lower at the caudal extremity of the POHA than in the rest of the region. Dopamine is evenly distributed throughout the region with the exception of the rostral extremity between A5-A4 where the concentration is approximately four times greater.

Sagittal slices (Figure 3b)

In the lateral direction, the distribution of 5-HT contrasts sharply with that of noradrenaline for,

Table 1 The weight of the rabbit preoptic hypothalamic area (POHA) derived by various methods.

POHA dissected as:		*Slice weight (mg)*	*Total weight of POHA area (mg)*
1. Whole area		–	215 ± 4.3
2. Frontal slices	A54	20.0 ± 1.8	
	A43	21.3 ± 1.7	
	A32	24.8 ± 1.2	
	A21	27.3 ± 1.4	
	A10	29.3 ± 1.4	
	P01	29.5 ± 1.5	
	P12	28.2 ± 1.1	
	P23	26.5 ± 1.3	207.8 ± 9.1
3. Sagittal slices	L32	24.3 ± 3.9	
	L21	35.7 ± 1.6	
	L10	41.7 ± 3.0	
	R01	37.7 ± 1.4	
	R12	35.5 ± 1.9	
	R23	30.7 ± 1.1	205.5 ± 9.4
4. Horizontal slices	Below V5	6.8 ± 2.4	
	V54	30.3 ± 4.0	
	V43	45.7 ± 1.9	
	V32	46.3 ± 1.9	
	V21	47.7 ± 1.9	
	V10	44.2 ± 1.8	221.0 ± 5.6

Weights are mean ± s.e. derived from 6-8 animals.

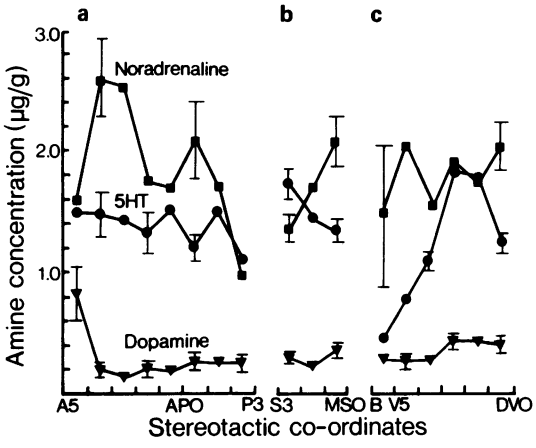

Fig. 3 The distribution of noradrenaline (■), dopamine (▼) and 5-hydroxytryptamine (5HT) (●) in (a) frontal slices; (b) sagittal slices; (c) horizontal slices derived from the preoptic hypothalamic area of rabbit brain. Values are means ±s.e. obtained from 6-8 animals.

while the latter amine appears concentrated in the structures immediately adjacent to the mid-sagittal line (i.e. between MSO and S1 (where S denotes sagittal and S1 corresponds to L1 or R1 in Fig. 2)), 5-HT is concentrated towards the lateral borders of the region (i.e. towards S3). Dopamine is evenly distributed throughout the sagittal planes.

Horizontal slices (Figure 3c)

The distribution of 5-HT in a horizontal direction throughout the POHA presents a definite pattern with an area of low concentration at the base of the brain. 5-HT concentration then rises to peak between V3 and V1 before falling slightly in the most dorsal slice. Noradrenaline appears evenly distributed throughout the area but the concentration of this amine changes rapidly in the area between V5 and the base of the brain as evidenced by the large standard error on the most ventral value. Dopamine is evenly distributed throughout the horizontal slices.

Statistical analysis

To ascertain whether the distribution of amines described above was due to true intra-hypothalamic differences or whether the observed variation was merely due to inter-hypothalamic differences, the data was subjected to an analysis of variance. Comparison was made between the concentration of amine in individual slices rather than the absolute amount of amine per slice. In this way allowance was made for variation in slice weight. The results showed that for noradrenaline the concentration of the amine does vary significantly between slices cut in both frontal ($P < 0.001$) and sagittal ($P < 0.001$) planes but not between slices cut in a horizontal plane. Similarly, the concentration of 5-HT varied significantly between slices cut in the horizontal ($P < 0.001$) and sagittal ($P < 0.001$) planes; but not between slices cut in the frontal plane. Dopamine varied significantly only between frontal slices ($P < 0.01$), reflecting the large concentration found in the most rostral slice A54 when compared with the rest of the region.

Correlation between amine concentration and anatomical features

Sawyer *et al.* (1954), Fifkova & Marsaka (1962), Monnier & Gangloff (1961) and Ridge (1964) have all produced atlases of rabbit brain. In order to consider the possible significance of the different distribution patterns for the three amines described above, it is essential to take account of the anatomical structures contained in tissue slices obtained by stereotactic dissection. Figures 4 and 5 are drawings reconstructed from the atlases of

Table 2 The amine content of the rabbit preoptic hypothalamic area.

	Amine concentration (µg/g)		
	Noradrenaline	*Dopamine*	*5-HT*
1. Area dissected as a whole	1.61 ± 0.06	0.32 ± 0.03	1.38 ± 0.15
2. Area dissected as frontal slices	1.80 ± 0.25	0.28 ± 0.06	1.38 ± 0.10
3. Area dissected as sagittal slices	1.73 ± 0.13	0.31 ± 0.03	1.48 ± 0.08
4. Area dissected as horizontal slices	1.80 ± 0.18	0.38 ± 0.05	1.33 ± 0.14

Values are mean ± s.e. expressed as µg/g fresh weight obtained from 6-13 animals.

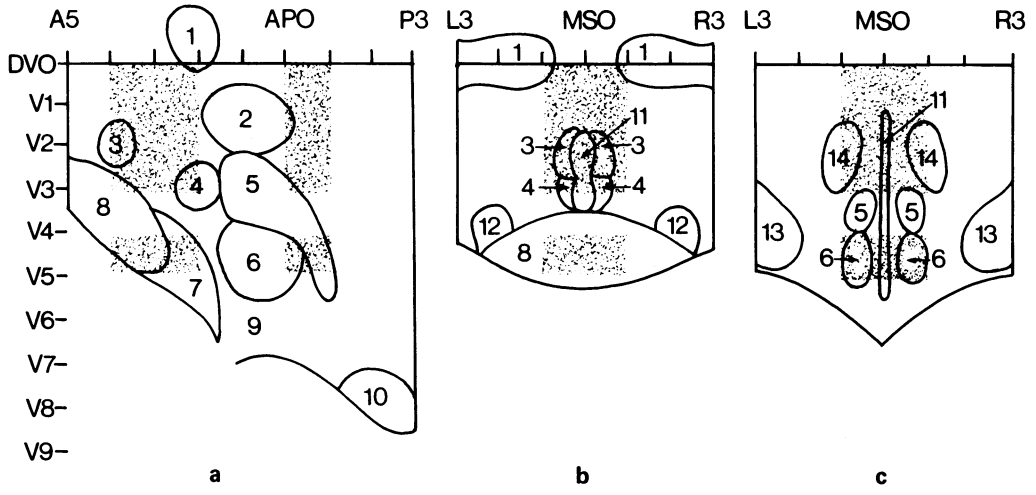

Fig. 4 Profiles of the rabbit preoptic hypothalamic area in; (a) the mid-sagittal plane; (b) frontal plane at A3; (c) frontal plane at P0.5. The axes represent the stereotactic co-ordinates. Key to numbered structures occurring in both Figure 4 and 5. 1, Anterior commissure; 2, Paraventricular n.; 3, Preoptic n.; 4, Suprachiasmatic n.; 5, Dorsomedial hypothalamic n.; 6, Ventromedial hypothalamic n.; 7, Diffuse supraoptic n.; 8, Optic chiasma; 9, Arcuate n.; 10, Mammillary body; 11, Third ventricle; 12, supraoptic n.; 13, Optic tract; 14, Fornix; 15, Reticular n.; stippled area represents: noradrenaline > 2.0 μg/g.

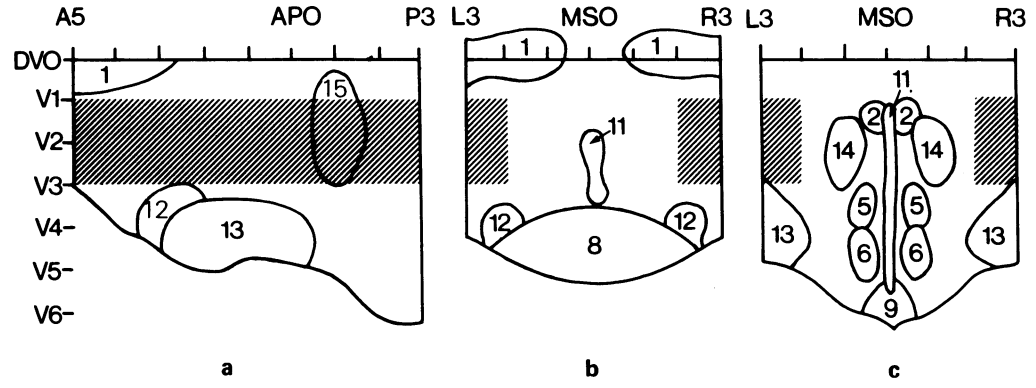

Fig. 5 Profiles of the rabbit preoptic hypothalamic area in (a) sagittal plane at S2.5; (b) frontal plane at A3 and; (c) frontal plane at APO. The axes represent the stereotactic co-ordinates. Key as in Figure 4. Hatched area represents: 5-HT > 1.6 μg/g.

Sawyer *et al.* (1954) and Ridge (1964) to illustrate some of the features contained in various slices. Figure 4a is a profile of the POHA in the mid-sagittal plane and anatomical features which are located between the mid-sagittal line and a plane 1 mm lateral to the mid-line have been superimposed on it. Similarly, Fig. 4b represents a frontal profile of the POHA as it occurs at A3 with the anatomical features which occur between A4 and A2 superimposed on it, and Fig. 4c represents a frontal profile at P0.5 with structures occurring between APO and P1 superimposed.

In Fig. 3b noradrenaline is shown to be concentrated close to the mid-sagittal line i.e. it could be associated with any of the structures depicted in Figure 4a. Consideration of the distribution of noradrenaline in the rostro-caudal direction (Fig. 3a) shows the amine to be con-

centrated between A4-A2 and APO-P1. Contained between A4 and A2 in the area adjacent to the mid-line are the preoptic nuclei, the area beneath the anterior commissure, the suprachiasmatic nucleus, part of the optic chiasma and the diffuse supraoptic nucleus, whilst between APO and P1, is located the area immediately caudal to the paraventricular nucleus and the caudal extremities of both the dorsomedial and ventromedial nuclei. Information derived from the horizontal slices does not permit further definition of the areas in the POHA where noradrenaline is located. The shaded areas in Fig. 4 represent areas of the POHA where the concentration of noradrenaline is greater than 2.0 μg/g.

Similarly, analysis of the distribution of 5-HT within the POHA shows it to be concentrated in the shaded areas of Fig. 5 where shading represents a 5-HT concentration greater than 1.6 μg/g. Dopamine appears evenly distributed throughout the POHA in all three planes and an analysis of the type presented for noradrenaline and 5-HT does not produce sharply defined areas of dopamine concentration.

Discussion

The distribution of noradrenaline, dopamine and 5-HT within the preoptic hypothalamic area of the rabbit brain has been determined by the method of stereotactic dissection followed by fluorimetric assay. The results show that noradrenaline is concentrated around the mid-sagittal line, particularly in the region between the optic chiasma and the anterior commissure and also in the region immediately caudal to the main hypothalamic nuclei. By contrast, 5-HT is concentrated towards the lateral borders of the hypothalamus in the vicinity of the supraoptic and reticular nuclei. Dopamine is evenly distributed throughout the preoptic hypothalamic region.

Prior to this study, description of the distribution pattern for these amines within anatomically complex areas of the CNS had relied on the histochemical approach introduced by Falck (1962). Using the histochemical approach, various groups of investigators (Carlsson, Falck, Hillarp & Torp, 1962; Fuxe, 1965; Andén *et al.*, 1966) reported that in the rat hypothalamus, noradrenaline was concentrated in: (a) the preoptic region just below the anterior commissure; (b) the supraoptic nuclei; and (c) the paraventricular nuclei.

Owman & Falck (1965) reported an area of high noradrenaline concentration immediately posterior to the paraventricular nuclei, but little noradrenaline in the posterior hypothalamus. Because 5-HT-containing fibres were finer and the fluorescence produced decayed rapidly on exposure to ultraviolet radiation, these neurones have proved more difficult to locate (Fuxe, 1965). Nevertheless, 5-HT-containing fibres have been located in the lateral hypothalamus (Fuxe, 1965; Andén, Dahlström, Fuxe & Larsson, 1965). Dopamine was reported by Fuxe (1965) to be concentrated predominantly in the neostriatum and the tuberculum olfactorium with lesser amounts in the external layer of the median eminence.

Thus, results obtained by histochemical techniques agree well with those obtained by the present method. In both cases the highest concentrations of noradrenaline were found close to the midline in the preoptic region, while lower concentrations were located caudal to the paraventricular nucleus: by contrast, 5-HT appeared to be concentrated in the lateral hypothalamus. This correlation between the two methods is reassuring. It is not possible with the present method to define the location of the amines with the same degree of precision as with the histochemical method because it is not practicable to cut slices thinner than 0.5 mm. On the other hand, the method using stereotactic dissection possesses certain advantages over the histochemical method:

(a) It is able to provide quantitative data, whereas histochemical fluorescence is only able to provide semi-quantitative data by the use of rating scales (e.g. Fuxe, 1965) although attempts are being made to remedy this deficiency (Gillespie, Hamilton & Hosie, 1970).

(b) With the present method, slices are subjected to specific extraction and estimation procedures, so that positive differentiation can be made between the various amines. With the histochemical methods, it is necessary to differentiate visually between catecholamine fluorescence at 480 nm and 5-HT fluorescence at 530 nm, and furthermore to confirm amine identity by means of selective depletion with drugs.

(c) By means of the stereotactic dissection technique, it is possible to map out the distribution of compounds for which histochemical techniques are not available, e.g. cytochrome oxidase (Ridge, 1967).

(d) The slices which are derived from fresh tissue by the present method, can be subjected to subsequent biochemical experiment: this is not possible with histochemical techniques.

(e) By the use of appropriate homogenization conditions, complete release of amines can be assured (Green, 1962), so that the method does not rely on the debatable assumption (Van Orden III, Schaefer, Burke & Lodoen, 1970), inherent in the histochemical method, that all of the tissue amines are accessible to the reacting chemicals.

Detailed statistical analysis of the results

obtained from the present experiments, which attempted to correlate the amines with individual anatomical structures, was not successful, probably because it is not possible to ascertain how each amine is distributed within an individual slice. Even if methods were derived to produce thinner slices of brain tissue, the present analytical methods are not sufficiently sensitive to detect the smaller quantities of amines which would be present.

I wish to thank Dr J.W. Ridge for providing me with training in the technique of stereotactic dissection and Professor J.W. Thompson for much valuable advice and encouragement during the course of this work.

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