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THE ORIGIN OF ACETYLCHOLINE APPEARING IN THE EFFLUENT OF PERFUSED CEREBRAL VENTRICLES OF THE CAT

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On perfusion of the cerebral ventricles of anaesthetized cats with an anticholinesterase, acetylcholine appears in the effluent collected from the cannulated aqueduct (Bhattacharya & Feldberg, 1958). The present experiments have been undertaken to find out from which part, or parts, of the ventricular system this acetylcholine originates.

Methods are now available for perfusing drugs through a lateral ventricle, through its anterior or inferior horn, or through the third ventricle or parts of it, while the remainder of the ventricular system is perfused with artificial c.s.f. (Carmichael, Feldberg & Fleischhauer, 1964). These methods of regional perfusion have been developed in order to make it possible to determine the site, or sites, of action of drugs when introduced into the cerebral ventricles. They may, however, also be adopted for determining the site of release of a substance into the cerebral ventricles provided its appearance is dependent on the presence of a drug in the perfusing fluid as, for instance, the presence of an anticholinesterase is required for the appearance of acetylcholine.

The acetylcholine which appears in the effluent on perfusion of the cerebral ventricles with an anticholinesterase, may enter the perfusing fluid in the lateral ventricle, its anterior and inferior horn, or in the dorsal and ventral half of the third ventricle. The results obtained with the methods of regional perfusion show that the acetylcholine enters the perfusion fluid in all these regions, but in greatly varying amounts.

METHODS

Cats of both sexes weighing between 2·3 and 2·9 kg were anaesthetized with chloralose (60–80 mg/kg) injected into the cannulated left femoral vein. To allow cannulation of the vein anaesthesia was induced with ethyl chloride and ether. The trachea was cannulated.

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With the cat lying on its belly the head was fixed to the ear bars and mouth-piece of a head holder similar to that of the Horsley–Clarke stereotaxic instrument.

To perfuse different parts of the cerebral ventricles with neostigmine, the method of multiple cannulation of the ventricular spaces was used. For this purpose several cannulae are inserted, each into a different part of the ventricular system. All cannulae, except one acting as the outflow, are attached to separate injectors and serve as inflows. One, or at most two, deliver neostigmine methyl bromide in a concentration of 1/50,000 and at a rate of 0·1 ml./min, and the others deliver artificial cerebrospinal fluid (c.s.f.) at a rate of 0·05 ml./min. The method is based on the principle of denying drugs access to those parts of the ventricular systems cannulated and perfused with artificial c.s.f. The details are the same as described by Carmichael *et al.* (1964). The artificial c.s.f. used for perfusion and for dilution of the neostigmine is that described by Merlis (1940). Its composition is (g/l.): NaCl, 8·1; KCl, 0·25; $CaCl_2$, 0·14; $MgCl_2$, 0·11; $NaHCO_3$, 1·76; Na_2HPO_4, 0·07; $CO(NH_2)_2$, 0·13; and glucose, 0·61.

For perfusion of one lateral ventricle (the left) with neostigmine, three cannulae were inserted into this ventricle, one into the anterior horn, one into the inferior horn, and the third into the body of the ventricle. The neostigmine was delivered through the anterior and inferior horn cannulae, and the cannula in the body served as outflow. To prevent the neostigmine entering the right lateral and the third ventricle artificial c.s.f. was perfused through a cannula implanted into the right lateral ventricle, and, in retrograde direction, through a cannula implanted in the aqueduct.

For perfusion with neostigmine of either the anterior or the inferior horn of the left lateral ventricle, the arrangement of cannulation was the same as for the perfusion of neostigmine through the entire lateral ventricle, but the neostigmine was delivered through only one cannula, either the anterior or the inferior horn cannula, the other delivering artificial c.s.f. In several experiments, instead of perfusing artificial c.s.f. in a retrograde direction through the third ventricle, the simplified modification of keeping the aqueductal cannula closed during the perfusion was used.

For perfusion of the third ventricle a cannula delivering the neostigmine was inserted into this ventricle, in the mid line, anterior to the massa intermedia, and the outflow was from the cannulated aqueduct. Neostigmine was prevented from entering the lateral ventricles by simultaneously perfusing them with artificial c.s.f. through implanted cannulae. If the opening of the cannula inserted into the third ventricle lies below the massa intermedia, the perfusion of neostigmine is often restricted to the part of the ventricle lying ventral to the massa, the walls of this part of the ventricle being formed by the hypothalamus. This happened in three experiments as was verified by the fact that these regions of the walls of the third ventricle were stained with bromophenol blue, substituted for neostigmine at the end of the perfusion.

The effluent from the perfused ventricles was collected in half-hour samples and assayed on the eserinized leech muscle against acetylcholine chloride. All values of acetylcholine refer to the salt.

RESULTS

The results are summarized in Tables 1–5; they give the output of acetylcholine in nanograms per minute of successive half-hour samples of effluent collected from the cerebral ventricles perfused with neostigmine. In control experiments in which the cerebral ventricles were perfused without neostigmine, no acetylcholine was detected in the effluent.

Table 1 shows the acetylcholine output in three experiments in which neostigmine is perfused through one (the left) lateral ventricle, but prevented from entering the third, whereas Table 2 gives the acetylcholine output in five experiments in which the reverse procedure is adopted and

the neostigmine is perfused through the third ventricle only. The experiments show that with both procedures acetylcholine appears in the effluent. However, much more acetylcholine is released from structures lining the lateral ventricle since on its perfusion with neostigmine the mean acetylcholine output rises from 3·6 ng/min during the first half-hour to 8·6 ng/min during the third hour, while on perfusion of the third ventricle

TABLE 1. Acetylcholine output in the effluent of successive half-hour samples during perfusion of the left lateral ventricle with neostigmine

Acetylcholine (ng/min)			
Expt.			Mean
1	2	3	value
4·2	—	3·0	3·6
7·3	3·8	3·2	4·7
5·0	3·4	3·6	4·0
8·1	4·2	3·0	5·0
6·2	5·8	4·4	5·3
11·8	7·8	5·2	8·6
(5·0)*	(2·5)*	(4·2)*	(2·9)*
		(2·2)*	(2·2)*

* Anaesthesia deepened.

TABLE 2. Acetylcholine output in the effluent of successive half-hour samples during perfusion of the third ventricle with neostigmine

Acetylcholine (ng/min)					
Expt.					Mean
1	2	3	4	5	value
0·3	0·9	0·8	0·8	1·1	0·8
0·3	0·9	1·0	0·8	1·1	0·8
0·3	0·9	0·9	1·0	1·1	0·8
0·3	1·1	1·0	1·0	1·0	0·9
0·3	1·0	0·9	1·0	1·0	0·8
0·3	1·1	0·8	1·0	1·2	0·8
0·5	1·3	1·1	1·0	1·2	1·0
0·7	1·1	0·9	1·0	1·2	1·0

the mean output is 0·8 ng/min in the first half-hour and rises to 1 ng/min only. There is thus not only from the beginning a lower output from the walls of the third ventricle, but in addition there is on continued perfusion scarcely any increase, as occurs on perfusion of neostigmine through the lateral ventricle.

From the results given in Tables 3 and 4, it is seen that the structures lining both the anterior and the inferior horn of the lateral ventricle contribute to the high acetylcholine output obtained on perfusion of the entire lateral ventricle. The greater contribution, however, comes from structures lining the anterior part; further, the acetylcholine output in-

creases on continued perfusion of the anterior part but not of the inferior horn.

The output of acetylcholine on perfusion of neostigmine through the third ventricle arises mainly from its dorsal half. This is evident when the output, on perfusion of neostigmine through the entire third ventricle (Table 2), is compared with that on perfusion of the part which lies ventral to the massa intermedia, the walls of this part being the hypothalamus

TABLE 3. Acetylcholine output in the effluent of successive half-hour samples during perfusion of the anterior horn of the left lateral ventricle with neostigmine

Acetylcholine (ng/min)

Expt.					Mean
1	2	3	4	5	value
2·2	2·1	2·9	2·3	2·3	2·5
2·3	2·3	4·4	2·5	2·4	2·7
2·3	2·3	3·1	2·5	2·8	2·6
1·7	2·8	4·5	3·5	2·4	3·0
3·5	2·7	3·0	3·8	2·8	3·1
3·2	6·0	5·0	4·1	3·4	4·3
3·2	5·0	4·2	4·7	4·2	5·0
3·2	8·8	4·3	4·8	5·5	5·3

TABLE 4. Acetylcholine output in the effluent of successive half-hour samples during perfusion of the inferior horn of the left lateral ventricle with neostigmine

Acetylcholine (ng/min)

Expt.					Mean
1	2	3	4	5	value
1·1	1·1	0·9	0·7	0·9	0·9
1·8	1·1	1·0	0·7	0·9	1·1
1·1	1·4	1·0	1·1	0·8	1·1
1·8	1·4	1·0	1·1	0·8	1·2
1·1	1·1	1·0	1·1	0·8	1·0
0·9	1·2	1·1	1·1	0·9	1·0
0·8	1·0	1·2	1·1	0·9	1·0
0·9	0·9	1·4	1·1	0·9	1·0

TABLE 5. Acetylcholine output in the effluent of successive half-hour samples during perfusion of the ventral part of the third ventricle with neostigmine

Acetylcholine (ng/min)

Expt.			Mean
1	2	3	value
0·3	0·2	0·3	0·3
0·3	0·2	0·3	0·3
0·3	0·2	0·3	0·3
0·4	0·2	0·4	0·3
0·4	0·2	0·3	0·3
0·4	0·3	0·3	0·3
0·3	0·3	0·3	0·3
0·3	0·3	0·3	0·3

(Table 5). The output is about a third of that obtained on perfusion of the entire third ventricle.

Deepening of anaesthesia. The acetylcholine output from the perfused cerebral ventricles depends on the depth of anaesthesia. If, in the course of a prolonged perfusion of the lateral ventricle with neostigmine, anaesthesia is deepened by additional intravenous chloralose (40 mg/kg) after the acetylcholine output has reached a high level, the output falls. This is illustrated in Table 1.

DISCUSSION

The aim of the present experiments has been to find out the site, or sites, of origin of the acetylcholine that appears in the effluent during perfusion of the cerebral ventricles with an anticholinesterase, and the method used made it possible to determine where, in the ventricular system, the acetylcholine enters the perfusing fluid. This was found to take place in all parts examined. The acetylcholine enters the perfusion fluid in the anterior and inferior horn of the lateral ventricle, and in the dorsal and ventral half of the third ventricle. But the amounts vary. The largest contribution is made into the anterior horn of the lateral and the smallest into the ventral half of the third ventricle.

The ependyma lining the ventricular spaces can scarcely be considered to be the tissue from which the acetylcholine arises. The most likely, and probably the sole source, is in the layers of grey matter which border the lumen of the ventricles and contain acetylcholine as well as the enzyme for its synthesis, choline acetylase. We must assume that some of the acetylcholine continuously synthesized and released in this grey matter escapes into the ventricular spaces if its destruction is prevented by an anticholinesterase penetrating the brain tissue from the perfused ventricles.

On this assumption the differences in the amounts of acetylcholine added to the perfusing fluid in the different parts of the ventricular system can best be explained by variations in the following three factors: (1) The area of grey matter lining the lumen, (2) the thickness of the grey matter, and (3) the concentration of acetylcholine and choline acetylase in the grey matter.

Not only the surface area but also the thickness of the grey matter will have an effect because of the depth of penetration of the anticholinesterase. During prolonged perfusion such widely different substances as histamine and bromophenol blue may penetrate some grey matter to a depth of a few millimetres from the ventricular lumen (Feldberg & Fleischhauer, 1960), and the same may therefore happen with neostigmine, the anticholinesterase used in the present perfusions.

The concentration of acetylcholine and choline acetylase in grey matter probably depends on the density of cholinergic neurones. MacIntosh (1941) determined the content of acetylcholine in various parts of the brain and found great variations between different regions of grey matter. Similar determinations have been made for the choline acetylase in the brain of the dog by Feldberg & Vogt (1948) and in the brain of several mammals, including the cat, by Hebb & Silver (1956). In general it may be said that the concentrations of the two substances run parallel in the different parts of the brain.

The finding that the largest contribution of acetylcholine is made in the anterior horn of the lateral ventricle is easily explained by the fact that the caudate nucleus bulges into the horn and presents a large surface area in its lateral wall. This nucleus has the highest acetylcholine and choline acetylase content in the brain. According to Hebb & Silver its choline acetylase activity in cats, expressed in milligrams of acetylcholine synthesized in 1 hr per gram tissue, is between 7·5 and 11·5 mg. The acetylcholine entering the anterior horn probably originates mainly from the caudate nucleus. Recently Mitchell & Szerb (1962), with the technique of the 'push-pull' cannulae of Gaddum (1961), have shown that acetylcholine is released into fluid irrigating small areas of this nucleus and that this release increases on direct or indirect stimulation.

The caudate nucleus is a rather thick mass of grey matter. By analogy with the penetration of bromophenol blue and histamine from the ventricular lumen we may expect the neostigmine to penetrate the caudate nucleus more deeply than any other grey mass. This is probably the reason why the acetylcholine content in the effluent from the anterior horn usually increases during prolonged perfusion, whereas no similar increase occurs in the effluent from other parts of the ventricular system.

Some of the acetylcholine entering the fluid perfusing the anterior horn may also come from the olfactory tract and bulb as they contain appreciable amounts of acetylcholine and choline acetylase. The values given by Hebb & Silver for the choline acetylase activity of the tract and bulb in the cat are 3·6 and 2·2 mg ACh/g/hr. Again by analogy with the depth of penetration of bromophenol blue we may expect relatively deep penetration of neostigmine from the olfactory recess. No data are available about the acetylcholine and choline acetylase content of the septum which forms part of the medial wall of the anterior horn. Whether an additional contribution of acetylcholine is made from this structure therefore remains an open question.

The acetylcholine entering the fluid perfusing the inferior horn is probably derived mainly from the hippocampus and amygdala which form a large surface area in this horn and contain acetylcholine and choline

acetylase, but in lower concentrations than found in the caudate nucleus. The values obtained by Hebb & Silver in cats are 3·5 mg ACh/g/hr for the choline acetylase activity of hippocampus and 2·5–5·5 mg ACh/g/hr for amygdala. The fact that the acetylcholine in the effluent from the inferior horn did not increase during prolonged perfusion with neostigmine, as does occur in the effluent from the anterior horn, may be due to neostigmine penetrating the hippocampus and amygdala less readily than the caudate nucleus. Bromophenol blue, for instance, did not penetrate the hippocampus as deeply as the caudate nucleus, and this was explained by the fact that the hippocampus is covered by the alveus, a thin layer of white matter, which the dye penetrates less readily than grey matter. Some of the acetylcholine found in the effluent from the inferior horn may also have been released from the grey matter of the pyriform lobe which probably contains acetylcholine and choline acetylase, and by analogy with bromophenol blue we would expect the neostigmine to penetrate this lobe from the inferior horn.

The two structures responsible for the acetylcholine which enters the perfusion fluid in the third ventricle would appear to be the thalamus and hypothalamus. The walls of the ventral half are formed by the hypothalamus, the acetylcholine and choline acetylase content of which is low, lower than that of the thalamus. This would explain why so little acetylcholine appeared in the effluent when the perfusion with neostigmine was limited to the ventral half of the third ventricle. Hebb & Silver give for the choline acetylase activity of the cat's hypothalamus a value as low as 0·25 mg/ACh/g/hr which is about seven times lower than that of the thalamus (1·7–1·75 mg/ACh/g/hr) and 28–46 times lower than that of the caudate nucleus. In addition the surface area of the hypothalamus in the walls of the ventral part of the third ventricle is relatively small and the grey matter forms a layer thinner than the caudate nucleus in the wall of the anterior horn. In fact not all the acetylcholine collected from the ventral half of the third ventricle may be derived from the hypothalamus; some may be released from the lower rim of the massa intermedia which we may expect, from analogy with bromophenol blue, to have been penetrated by the neostigmine. A further small contribution may also be made by acetylcholine derived from the central grey matter around the perfused rostral end of the aqueduct.

The fact that the hypothalamus contributes only a small percentage to the total acetylcholine content appearing in the effluent obtained from perfusion with an anticholinesterase of an entire lateral and third ventricle may explain why Hilton & Schain (1961) did not detect an increase in the acetylcholine output when they stimulated the hypothalamus electrically through implanted electrodes. Even if the release of acetylcholine from

the hypothalamus had doubled during stimulation, this increase would hardly have been detectable in their experiments. To demonstrate an increase the ventral half of the third ventricle alone would have to be perfused with the anticholinesterase.

The finding that deepening of anaesthesia by intravenous chloralose reduces the acetylcholine content in the effluent collected from a lateral ventricle perfused with neostigmine shows that the release of acetylcholine is reduced from either all or some of the structures which contribute to the total acetylcholine appearing in the effluent. As it has been shown that the caudate nucleus contributes most to the acetylcholine content of the effluent it is difficult to exclude an effect of chloralose on this structure. Chloralose would thus have the same effect on this basal ganglion as on the cerebral cortex because chloralose also reduces the release of acetylcholine in the cerebral cortex. This was first found by MacIntosh & Oborin (1953) and has recently been confirmed by Mitchell (1963), who attributes the effect to depression of high frequency cortical activity. The reduction in the release of acetylcholine from the caudate nucleus is therefore most likely the result of chloralose depressing nervous activity in this grey matter involving cholinergic neurones.

The present results presuppose a two-directional active transport of substances through the layer of ependyma and within the brain tissue. No acetylcholine could have appeared in the perfusing fluid if the neostigmine had not first passed through the ependyma and into the adjacent grey matter, there to prevent the destruction of the released acetylcholine. But in order to appear in the ventricular fluid the undestroyed acetylcholine must then pass from its site of release, in the opposite direction, to and through the ependyma. Simultaneous two-directional transport of substances must therefore occur through the ependyma and within the adjacent brain tissue where the neuroglia would appear to be the most likely structure concerned with the transport.

SUMMARY

1. In cats anaesthetized with chloralose methods of regional perfusion of the cerebral ventricles with neostigmine have been used to determine the site of origin of the acetylcholine which appears in the effluent of the cerebral ventricles perfused with artificial cerebrospinal fluid containing neostigmine.

2. The acetylcholine enters the perfusing fluid in the anterior and inferior horn of the lateral ventricle as well as in the dorsal and ventral part of the third ventricle, but in varying amounts.

3. The greatest amount of acetylcholine comes from structures lining

the anterior horn of the lateral ventricle, namely, from the caudate nucleus, the olfactory grey matter and perhaps from the septum.

4. The smallest amount of acetylcholine comes from structures lining the ventral half of the third ventricle, the walls of which contain the nuclei of the hypothalamus.

5. The differences in the amounts of acetylcholine entering the perfusion fluid in the different parts of the ventricular system are accounted for by variations in area and thickness of the grey matter lining the lumen, and particularly in the acetylcholine and choline acetylase concentration of this grey matter.

6. Deepening the chloralose anaesthesia reduces the amount of acetylcholine entering the perfused lateral ventricle.

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