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Effects of Calcium on the Release of Serotonin from Isolated Sites Within the Diencephalon of the Cat[1]

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VEALE, W. L., R. D. MYERS AND D. B. BELESLIN. *Effects of calcium on the release of serotonin from isolated sites within the diencephalon of the cat.* PHARMAC. BIOCHEM. BEHAV. 1(3) 259–264, 1973.–In the unanesthetized cat, isolated sites in the hypothalamus and regions adjacent to this diencephalic structure were perfused with a Krebs solution by means of concentric push–pull cannulae. At 20 sites a substance with serotonin-like activity was released spontaneously at an average rate of 0.33 ± 0.05 ng/30 min perfusion interval. An anatomical mapping revealed that the sites of release of a substance with serotonin-like activity were distributed widely throughout the hypothalamus. When the Krebs solution contained an excess in calcium ions, the rate of release of the substance increased at 12 of 19 sites, decreased at 3 others and remained unchanged at 4 perfusion loci. In those regions in which the output of the substance with serotonin-like activity increased in comparison to its average rate of release, 2.6 mM excess calcium evoked an increase of 1.06 ± 0.28 ng/30 min, whereas 10.4 mM excess calcium in the perfusate caused an increase in the output of the substance to 1.65 ± 0.34 ng/30 min.

Push–pull perfusion Transmitter release in brain Brain perfusion Assay of neurohumoral substances

WHEN a solution containing an excess in concentration of a cation such as calcium, magnesium, potassium, or sodium is injected into brain tissue or into the cerebral ventricles of a conscious animal, various changes occur in body temperature, vasomotor tone, respiratory rate and the behavior of the animal [10, 14, 16, 35, 40]. Since it is known that certain ions may influence the liberation of acetylcholine, serotonin (5-HT) or norepinephrine from nerve terminals, it is possible that the physiological effects of an individual cation may be due, at least in part, to the release of a putative neurotransmitter [4, 7, 9, 17, 30, 39].

The hypothalamus, a region of the brain from which behavioral and vegetative responses can be elicited by chemical stimulation [27,29], is known to contain high concentrations of 5-HT [1,13]. When this area of the brain is perfused by means of push–pull cannulae with solutions containing an excess in sodium or calcium ions, the temperature of the animal changes markedly depending upon the site of the perfusion [30]. Recently, we have shown that the release of 5-HT, an amine in the anterior hypothalamus implicated in the control of body temperature, is enhanced when the perfusion solution contains an excess in calcium [33]. The purpose of the present experiments, in which the push–pull perfusion technique [25] was employed, was to determine: (1) the nature of the spontaneous release of 5-HT within the hypothalamus of the cat in terms of the magnitude and the anatomical specificity of the output; (2) whether or not the release of 5-HT from the hypothalamus of the cat is affected by altering the ionic composition of the perfusion fluid.

METHOD

Surgical Procedures

Sixteen adult cats of either sex, and weighing between 2.6 and 3.6 kg were anesthetized with pentobarbitone sodium (36 mg/kg) injected intraperitoneally. Following procedures described previously by Myers for implantation [25], surgery was carried out under aseptic precautions using the stereotaxic coordinate system of Jasper and Ajmone-Marsen [21]. An incision was made on midline, the aponeurosis reflected, and four holes were drilled in the calvarium bilaterally. After the dura mater had been incised, each of four 17 ga stainless steel guide tubes was lowered stereotaxically and fixed firmly to the skull with cranioplast cement. The tip of each guide rested four mm above the intended perfusion site, so that the area to be

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perfused would not be damaged by the permanently indwelling guide tube.

In order to maintain a sterile preparation and to protect the array of guide tubes, a polyethylene pedestal with a screw top was positioned around the four guides, then fixed to the skull with stainless steel screws and filled with cranioplast cement [27]. Postoperatively, penicillin was administered intramuscularly for 7 days, and an interval of at least one week always elapsed before the first experiment.

Procedure for Push–pull Perfusion

An isolated area of the hypothalamus was perfused according to methods described earlier [25,26]. The outer cannula which was cut of 20 ga stainless steel needle tubing served as the pull tube; the inner or push cannula was made of 27 ga needle tubing and inserted through the rubber diaphragm of the pull cannula so that it extended 0.8–1.0 mm beyond the tip of this outer cannula. Each of the cannulae was connected by polyethylene tubing to a calibrated push or pull syringe mounted on a multichannel, Harvard infusion-withdrawal pump.

To prepare the perfusion solutions, ion-exchange, glass-distilled water was used and the solution was autoclaved or passed through a sterilized milli-pore filter (0.22 μ). To simulate extracellular fluid [45], a modified Krebs solution [31,32] was used which contained Na, 143.0 mM; K, 5.9 mM; Ca, 2.6 mM; Cl, 127.8 mM; glucose, 5.6 mM; Mg, 1.2 mM; SO_4, 1.2 mM; H_2PO_4, 1.2 mM; and HCO_3, 25.0 mM. The glassware, syringes and tubing used in all experiments were also pyrogen free. The rate of flow through the push–pull cannulae was 50 μl/min and the duration of each perfusion was always 30 min. When bromophenol blue dye was substituted as a perfusate in order to verify the amount of tissue perfused, a sphere of tissue 1.0 mm in dia. was usually tinged with the stain [31].

During the course of a perfusion, the animal was unrestrained or held gently. The samples were accepted for assay only if the volume of the effluent matched exactly that of the inflow and if the perfusate was clear and devoid of tissue fragments. Each effluent was collected on ice and if not tested on the same day, was kept at −10°C until assayed. In order to avoid variations in the output of 5-HT due to circadian rhythms [38,41], the experiments were always done at the same time each morning after the colonic temperature had remained stable for at least one hour and after the cat had received its morning ration of commercial canned cat food. An interval of 2–4 days elapsed between the perfusion of any one site, and the sequence of perfusions with a control solution of a hypothalamic perfusate containing an excess of a specific ion was random.

Assay for 5-HT in Effluent

The content of 5-HT-like activity in each 30 min perfusion sample was determined by a sensitive biological assay according to the method of Vane [5,44]. A 6-cm strip cut from the rat stomach fundus was suspended in a 5 ml bath containing Krebs solution at 37°C which was bubbled constantly with a mixture of 95% O_2 and 5% CO_2. The stomach strip was sutured to a Brush strain-gauge transducer so that the isotonic contractions of the muscle could be registered on the Esterline-Angus potentiometric recorder. The sample of effluent from the hypothalamus of a 5-HT standard was added to the organ bath every 4–6 min and kept in the bath for 90 sec, during which time the inflow of the modified Krebs solution was turned off. Between contractions, the Krebs solution in the organ bath was continually renewed from the bottom and overflowed from the top.

The solutions of 5-HT used as the standards were always made up in the same ionic concentration as the fluid which was perfused within the hypothalamus of the cat. All values of 5-HT are expressed in terms of the creatinine phosphate salt of 5-HT. The contractile activity of a sample of the perfusate was considered to be due to 5-HT only if: (1) the contraction produced by the effluent was similar in magnitude and configuration to that produced by the 5-HT standard; and (2) either methysergide of BROM-LSD (BOL), specific antagonists of 5-HT, added to the bath in doses of 2–20 μg abolished or significantly reduced the contractions to a perfusate and standard equally [28].

Histological Verification of the Cannula Sites

After a series of experiments was concluded, the position of each perfusion site was verified by standard histological procedures [27]. The cat was killed with an overdose of pentobarbitone sodium, and 10% formol-saline was perfused through the thoracic aorta. The brain was cut at 30 μ in the coronal plane on a cryostat, and every fourth section was then stained for cells and fibers following a method modified after Kluver and Barrera [24].

RESULTS

Resting Release of 5-HT from the Diencephalon

In order to obtain an estimate of the anatomical distribution of sites at which a substance with serotonin-like activity was released spontaneously in the unanesthetized cat, push–pull perfusions were carried out at 48 different loci within the hypothalamus. Three to 6 perfusions were usually carried out in each cat, however, in one instance 12 perfusions were carried out at one site and the activity did not seem to be reduced. An analysis of the serotonin-like activity in the perfusates revealed two types of loci: (1) those at which this substance was released in amounts large enough to be detected by the bioassay; and (2) sites at which the substance was not released in detectable quantities. Sites where a substance with serotonergic activity was released were found to extend from the most rostral portion of the hypothalamus caudally to the mammillary region. Figure 1 presents the distribution of sites within the hypothalamus and contiguous diencephalic structures. From this mapping, it can be seen that serotonin-like activity was present in the perfusates collected at approximately 40 per cent of the sites examined. The loci of release, which averaged 0.33 ± 0.05 ng/30 min perfusion interval for all of the control perfusions, were distributed primarily around the third ventricle. This activity was not detected in perfusates collected from sites in the caudal mammillary region, areas bordering the nucleus reuniens of the thalamus, or in the most rostral and lateral regions of the preoptic area.

Excess Calcium and Other Ions and 5-HT Release

An analysis of the concentration of serotonin-like activity in the effluent collected after the hypothalamic

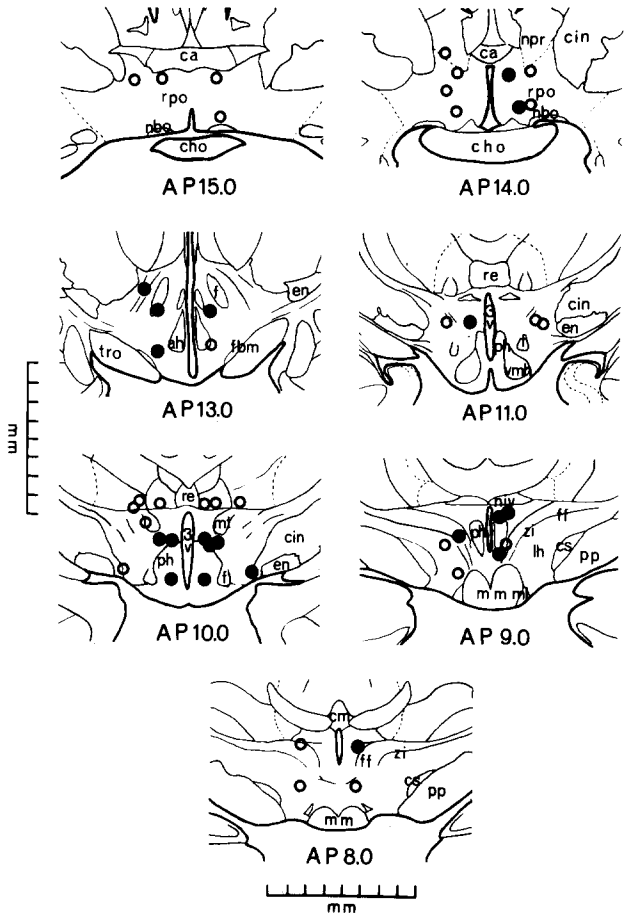

FIG. 1. Anatomical mapping of sites within the hypothalamus and its contiguous structures of the unanesthetized cat at which 5-HT was released (•) into a Krebs solution perfused at 50 μl/min for a 30 min interval. Sites at which 5-HT could not be detected by an assay usually sensitive to 0.1 to 0.3 ng/ml of the Krebs perfusate are indicated by the open circles (○). Anatomical abbreviations are: ah – anterior hypothalamic area; ca – anterior commissure; cho – optic chiasm; cin – internal capsule; cn – central medial nucleus of the thalamus; cs – subthalamic nucleus; db – diagonal band of Broca; en – endopeduncular nucleus; f – fornix; fbm – medial forebrain bundle; ff – fields of Forel; lh – lateral hypothalamic area; ml – lateral mammillary body; mt – mammillothalamic tract; nac – nucleus accumbens of the septum; nbo – supraoptic nucleus; niv – interventricular nucleus of the thalamus; npr – prothalamic nucleus; ph – posterior hypothalamic area; pp – cerebral peduncle; re – nucleus reuniens of the thalamus; rpo – pre-optic area; tro – optic tract; vmh – ventromedial nucleus; zi – zona incerta; 3V – third ventricle. The scales are in millimeters.

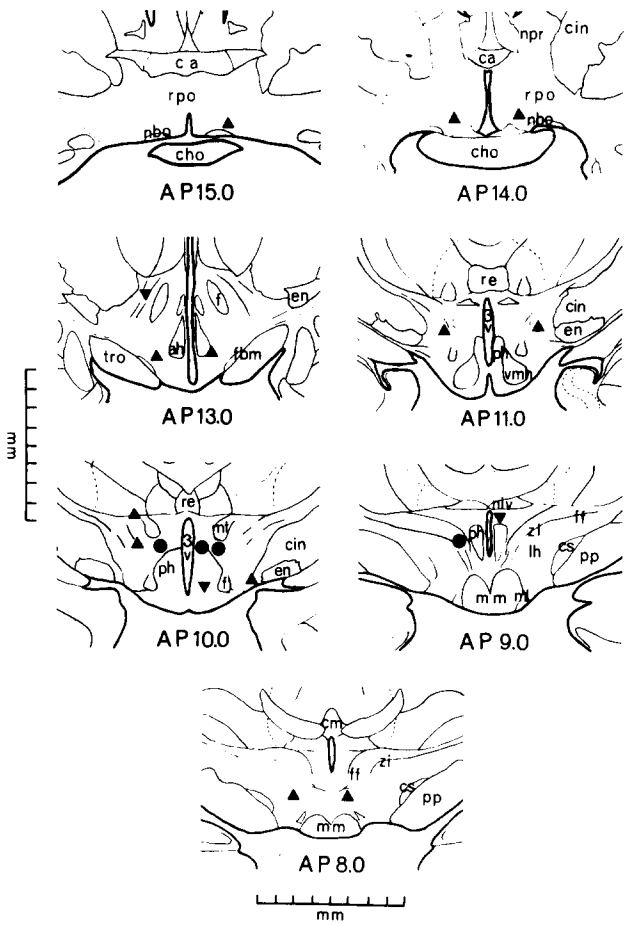

FIG. 2. Anatomical mapping of sites within the hypothalamus and several contiguous structures of the unanesthetized cat at which the rate of release of 5-HT increased (▲), decreased (▼) or remained unchanged (•), during a 30 min perfusion with a Krebs solution in which calcium was elevated by 2.6 or 10.4 mM above the normal value in the perfusate. Abbreviations are the same as in Fig. 1.

perfusion solution had been modified with excess calcium revealed three types of loci: (1) those at which the release of this activity was greater than that for the control perfusion at the same site; (2) sites at which the release of the serotonin-like substance was lower than the value obtained during a control perfusion; and, (3) those loci at which the release was unchanged. As illustrated in Fig. 2, an increase (▲) in the release of the substance with serotonin-like activity occurred at 12 of the 19 sites which were perfused with a solution containing calcium 2.6–10.4 mM in excess of the normal physiological concentration. The mean amount of this material in the effluent increased threefold from a control level of 0.27 ± 0.03 ng to 1.06 ± 0.28 ng/30 min perfusion interval, for all perfusions in which the perfusion solution contained twice the normal concentration of calcium. However, when the perfusion solution contained 5 times the normal physiological concentration of calcium, the mean output of this substance with serotonin-like activity was elevated fivefold, from a control level of 0.35 ± 0.07 to 1.65 ± 0.34 ng, during the same perfusion interval. At four perfusion sites, the output remained unchanged (○) and at three other sites, its rate of release declined (▼) to a mean level of 0.18 ± 0.04 ng/30 min as a result of the perfusion with the solution containing excess calcium ions. Figure 2 shows that the increase in the release of the serotonin-like material during the perfusion with excess calcium occurred at sites distributed throughout the hypothalamus from coronal planes AP 15.0 to AP 8.0. The sites at which the output declined were located in coronal planes AP 9.0, AP 10.0 and AP 13.0, whereas, the

sites at which no change in the output of a serotonergic substance transpired as a result of an excess in the calcium concentration, were located pincipally in coronal plane AP 10.0. Of special importance is the fact that the three differentiated kinds of sites were often found in close anatomical proximity to one another.

Table 1 presents mean values in ng/30 min perfusion intervals of the release of substance with serotonergic activity at 12 sites as shown in Figure 2 at which the concentration of calcium in the perfusate had been increased 2.6 or 10.4 mM above that in the Krebs solution. In general, the additional calcium caused a significant increase (see Table 1 for statistical tests) in the content of the amine in the perfusate. On the other hand, the results of the assays of the effluents obtained from sites within the hypothalamus which had been perfused with a solution containing sodium, potassium or magnesium in excess of their normal concentrations in the modified Krebs soltuion are also presented in Table 1. With regard to sodium, a 34.0 to 68.0 mM excess of this cation in the standard solution interfered with the bioassay. Usually the fundus strip relaxed and the contractile effect of 5-HT was blocked. When a very sensitive fundus strip was obtained, it was found that excess sodium in the perfusate did not enhance the release of a substance with serotonergic activity consistently, which was shown in seven such experiments. Potassium or magnesium perfused at corresponding sites in the hypothalamus in concentrations 2–5 times their normal value in the Krebs solution also did not evoke a consistent change in the content of the serotgonergic activity in this perfusate. Although there was considerable variation in the release of the substance with serotonin-like activity in response to both of these cations, potassium caused a slight reduction in 5-HT output in 7 of 10 experiments and an increased release only once.

It is possible that calcium ions might cause changes in the release not only of serotonin-like activity but also of an interfering substance. If this were the case, one would expect that the same material could also increase the assay response when potassium, sodium or magnesium was increased in the perfustate or when the modified Krebs solution alone was perfused. This of course did not occur; the contractions were increased in magnitude only during the assay of perfusates containing excess calcium. Further, the assay procedure which included the use of BOL and methysergide indicates strongly that the contractions of the fundus strip were due to 5-HT. In nine additional paired experiments involving three cats in which push–pull perfusion were carried out in the region of the lateral hypothalamus, approximately 1.0 ng/ml 5-HT was added to the modified Krebs perfusion solution. The 5-HT-like activity in the 9 control perfusions was estimated to be 0.92 ± 0.15 ng/ml whereas the following the addition of 10.4 mM Ca^{++} to the perfusion solution of 5-HT-like activity was 2.63 ± 1.0 ng/ml. The ability of these samples to contract the stomach fundus was prevented by the addition of methysergide as described in Method.

DISCUSSION

It is apparent that the ionic constituents of the extracellular fluid in the nervous system may affect or modify the presynaptic release of a transmitter substance. For example, calcium is essential for both the spontaneous and evoked release of acetylcholine at peripheral synapses including the ganglion and the neuromuscular junction [12,

TABLE 1

THE OUTPUT OF A SUBSTANCE WITH SEROTONIN-LIKE ACTIVITY FROM THE HYPOTHALAMIC SITES REPRESENTED IN FIGS. 1 AND 2 FOR BOTH CONTROL PERFUSIONS AND THE CORRESPONDING PERFUSIONS WITH EXCESS CALCIUM, SODIUM, POTASSIUM, AND MAGNESIUM. THE MEAN AND STANDARD ERROR FOR SEROTONERGIC ACTIVITY IS EXPRESSED AS 5-HT IN ng PER 30 MIN INTERVAL. THE RESULTS OF A *t*-TEST FOR DIFFERENCES ARE GIVEN IN THE RIGHT COLUMN

Krebs Control Perfusage = 0.27 ± 0.03	Krebs Plus 2.6 mM Ca^{++}	= 1.06 ± 0.28	$t = 2.78, df = 6; p<0.05$
Krebs Control Perfusate = 0.35 ± 0.07	Krebs Plus 10.4 mM Ca^{++}	= 1.65 ± 0.34	$t = 3.82, df = 9; p<0.01$
Krebs Control Perfusate = 0.41 ± 0.11	Krebs Plus 34.0 or 68.0 mM Na^{+}	= 0.55 ± 0.08	$t = 1.40$, N.S.
Krebs Control Perfusate = 0.47 ± 0.17	Krebs Plus 4.7 or 18.8 mM K^{+}	= 0.24 ± 0.05	$t = 1.27$, N.S.
Krebs Control Perfusate = 0.37 ± 0.09	Krebs Plus 4.8 mM Mg^{++}	= 0.33 ± 0.05	$t = 0.32$, N.S.

15, 18, 22, 23]. In the central nervous system, there is evidence which suggests that the release of several putative transmitters is calcium-dependent including norepinephrine [3,36], gamma-amino-butyric acid [20], acethylcholine [7, 17, 39], and possibly even dopamine [37].

In other work in which 11.0 mM excess calcium was added to the incubation medium, Chase *et al.*, [9] found that the release of 5-HT was not modified in rat brain slices. Nevertheless, in the present experiments, a region in the brainstem of the unanesthetized cat which is rich in endogenous 5-HT is highly sensitive to an alternation in the extracellular concentration of calcium. Inasmuch as the output of a serotonin-like substance is increased significantly ($p<0.01$) above the resting level in many of the sites perfused, when the calcium concentration was increased above that normally found in interstitial fluid [11,45], two important factors would seem to differentiate our results from those of Chase *et al.*, [9].

First, the hypothalamus is known to contain ascending monoaminergic fiber systems with a high density of 5-HT containing fibers [2]. In addition, Bedard *et al.*, [4] demonstrated that lesions to the nigra-striatal fiber system within the ventromedial tegmental area caused a marked depletion of the 5-HT in more rostral portions of the brainstem. In our experiments, areas of the cat's diencephalon were perfused in which a resting release of sertonin-like substance occurred presumably within areas in which a high endogenous concentration of 5-HT was available at the nerve endings of a given fiber system. In this connection, the anatomical pattern of the spontaneous release of this substance in the cat corresponds well with that observed in the unanesthetized monkey [6,28]. Second, the intact and unanesthetized preparation may provide a more representative picture of the neuronal activity, thus, transmitter release of this part of the brainstem than does isolated brain tissue. Roberts and Straughan [42] have shown that the use of an anesthetic may also affect rather profoundly the electrical activity of a single neurone in the cat in response to the iontophoretic application of 5-HT.

Since 7 of the 19 loci examined did not show an increase in the output of a serotonergic substance during perfusion with excess calcium, it is possible that if these sites were located in an area possessing a greater percentage of fibers than nerve endings. Thus, the synaptic activity of the site may not have been sampled. In several experiments in which serotonin-like activity was not detected in the effluent of a control perfusion, but an excess in the calcium concentration of the hypothalamic perfusate caused a release from the same site, the tip of the cannula may have been some distance from a concentration of serotonergic cells; however, during the perfusion with excess calcium, the output may have been sufficiently enhanced so that the substance was collected at the cannula tip.

When calcium is injected either into the cerebral ventricles [14], or directly into the brain tissue [10], sedation, a decrease in repiratory rate and drowsiness is produced. Over 10 years ago, it was suggested by Brodie and his colleagues [8] that the depressant action of reserpine and perhaps other hypnotic agents could be due to an increase in the release of 5-HT within the central nervous system. Since perfusion with excess calcium evoked these behavioral changes and other physiological responses, it is possible that a 5-HT mechanism may be involved in the mediation of these responses. Undoubtedly, 5-HT has a multiplicity of functions within the diencephalon at least based on experiments in which the indole amine is microinjected directly into the hyptohalamus in small doses [29,34]. Further, if the calcium in the perfusion medium would either diffuse or undergo transport to a locus some distance from the tip of the cannula, it is possible that an active release of 5-HT or other neurohumor, or combination of neurohumors could also cause a behavioral or physiological response at a more remote site. In any case, the fact that the release of a serotonin-like substance into the perfusion medium within certain isolated regions of the diencephalon is related to the local concentration of calcium lends further support to the role of 5-HT as a possible transmitter [43].

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