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In Vivo Determination of Endogenous Biogenic Amines in Rat Brain Using HPLC and Push-Pull Cannula[1,2]

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LOULLIS, C. C., J. N. HINGTGEN, P. A. SHEA AND M. H. APRISON. *In vivo determination of endogenous biogenic amines in rat brain using HPLC and push-pull cannula.* PHARMAC. BIOCHEM. BEHAV. 12(6) 959–963, 1980.— Combining the methods of push-pull cannulation with those of high performance liquid chromatography (HPLC), we have measured the content of a number of biogenic amines in the perfusate of freely moving rats. In an initial study, the lateral hypothalamus (LH) was chronically implanted with a push-pull cannula and was perfused with 0.9% NaCl. Fifteen minute samples were collected through the push-pull cannula (flow rate: 25 μl/min) and aliquots of 200 μl were injected into the HPLC without any extraction or prepurification procedure. Simultaneous determination of the levels of 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), norepinephrine (NE) and dopamine (DA) in the perfusate was accomplished by means of HPLC with electrochemical detection. The HPLC system utilized a C-18 reverse phase column coupled with a glassy carbon detector. Results indicate that this combination of push-pull perfusions and HPLC assay can provide a simple, rapid, and sensitive technique for the in vivo simultaneous determination of the compounds released in discrete brain areas. In preliminary studies in which these methods were used, 50 mg/kg D,L-5-HTP was injected subcutaneously (SC) into rats implanted with push-pull cannulae and working on a variable interval (VI 1) schedule of reinforcement. Increases in 5-HTP, 5-HT and 5-HIAA were measured during the period of behavioral depression following 5-HTP administration. This technique could provide a useful tool in the assessment of neurochemical changes in brain during ongoing steady-state behaviors or during the disruption of behavior following administration of drugs, precursors, or other perturbations.

Catecholamines Indoles High performance liquid chromatography Push-pull perfusions Schedule dependent behavior

IN previous studies from our laboratories, we have demonstrated that both the increases in levels of total 5-HT in specific brain areas as well as the increases in levels of 5-HT in nerve ending fractions were correlated with the disruption of food-reinforced behavior in pigeons and rats following administration of a number of serotonergic precursors and drugs [1–7, 11]. One limitation of the experimental procedures used in these studies is that trained animals must be killed during the period of atypical behavior to permit the measurement of the level of neurotransmitters. A method that could provide for the in vivo determination of neurochemical changes in specific regions of the brain during concomitant behavioral changes in operant responding would provide the investigator with a powerful tool, as well as additional valuable data, in the study of neurochemical-behavioral interactions. One such method employs the technique of push-pull cannula implanted in discrete areas of the brain of an animal working in a behavioral situation.

The push-pull cannula perfusion technique, as originally described [9,10], has been frequently employed in the study of neurotransmitter release [12, 15, 17, 18, 19]. Its appropriateness as a method to be used in combination with ongoing operant behavior has been aptly demonstrated [14,16]. In most of the previous studies in which a push-pull cannula has been implanted in trained rats, the lateral cerebral ventricles have been the areas perfused, following the injection of a radioactive labelled compound. Such procedures were necessary in order to provide adequate sensitivity of the neurochemical measures. Now, however, the recently developed procedures of high performance liquid chromatography (HPLC) are available and have the advantage of allowing endogenous levels of a number of neurotransmitters to be measured simultaneously in the same small sample [13]. This eliminates the need for radioactive labelling and permits collection of perfusate during short periods of time not only in ventricles but also in discrete brain areas.

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In this study, we have combined the push-pull cannula technique with the HPLC method to measure a number of biogenic amines in perfusates from the LH of freely moving rats during a food-reinforced operant schedule. In addition, to determine the suitability of this method for quantitatively measuring neurochemical changes in brain during one type of behavioral disruption, we injected 5-HTP (SC) into the rats and measured changes in perfusate levels of 5-HTP, 5-HT, 5-HIAA and DA during the period of behavioral depression following 5-HTP administration.

METHOD

Animals and Behavioral Apparatus

Three male Wistar rats, weighing 303, 310, and 293 g, were allowed to adapt to individual home cages and were kept on a constant light-dark cycle (light 0600–1800 hr). They were maintained on a food deprivation schedule (80% free-feeding weight) and were trained to press a lever for sweetened condensed milk on a variable interval one minute (VI 1) schedule of reinforcement. During daily VI 1 sessions, milk was presented to the responding rat on the average of 0.15 ml per minute. This schedule was found to be adequate to obtain stable responding (approximately 5 resp/min) for 2 hr sessions.

The operant conditioning apparatus consisted of a 17×21×17 cm Plexiglas box with a standard stainless steel rod grid floor. A lever was mounted 10 cm above the floor on the front chamber wall. A dipper mechanism for the delivery of the milk reinforcement is mounted in the middle of the same wall, to the left of the lever. A 3 cm hole in the top of the box permitted the perfusion tubing to remain attached to the rat as it was working on the VI schedule. Lever presses and reinforcements were recorded on counters and a cumulative recorder.

Surgery and Histology

Following behavioral training, animals were anesthetized with 3 cc/kg Equithesin (Jensen-Salsbery Labs). Each rat was implanted with a concentric push-pull cannula in the right lateral hypothalamus according to the predetermined De Groot [8] coordinates: AP −5.4, L −2.0 and V −2.5 mm from the interaural line. The inner cannula (30 ga stainless steel tubing) extended 0.5 mm beyond the end of the outer (22 ga) cannula. The cannulae were constructed in our Institute shops. Four stainless steel screws were used to attach the cannula to the skull and the implant was secured with acrylic dental cement. At least 10 days of post-operative recovery elapsed prior to re-establishing baseline responding on the VI schedule.

Following the completion of the experiment, each rat was perfused intracardially with 0.9% saline followed by 10% Formaline. The brains were removed, frozen and sectioned at 50 μm. Tissue was stained with cresyl violet for histological verification of the cannula tip placement.

Procedure

Following post-operative recovery animals were reduced to their post-operative weights and given additional daily VI sessions. On the day of the perfusion experiment, the rats were connected to the pump (Gilson minipuls II) through polyethylene tubing and placed in the operant conditioning chamber. Following a 5 min period of lever pressing, the pump was switched on. Saline (0.9%) was pumped through the inflow lines and the inner cannula to the perfusion site. Extracellular fluid was pumped through the outer cannula to the outflow lines. Samples were collected every 15 min into 3 ml conical tubes which were placed on ice and contained 1 μl of 0.1 N HCl. The animals were perfused for 105 to 120 min at the rate of 25 μl/min. Lighting in the room was kept to a minimum and directed away from the perfusion lines as much as possible. The total number of lever presses for each corresponding 15 min perfusion sample was recorded.

After the collection of two 15 min samples, the pump was switched off. The first sample was discarded as washout whereas the second served as a baseline sample. Following this procedure, the rats were taken out of the test chamber and disconnected from the pump. They were then injected SC as rapidly as possible with either 50 mg/kg D,L-5-HTP or an equal volume of 0.9% saline solution. The rats were then immediately connected to the pump, placed in the operant chamber and the pump was restarted. Thereafter the collection of 15 min perfusion samples and 15 min response rates resumed. At the end of the experimental session, the animals were disconnected from the pump, removed from the operant chamber and returned to their home cage. Initially two animals, f-250 and f-280, were injected with 0.9% saline in order to determine changes in the levels of biogenic amines over several 15 min periods. Subsequently, two animals, f-250 and f-252, were injected with 50 mg/kg D,L-5-HTP.

Assay of Biogenic Amines

Determinations of DA, 5-HTP, 5-HT and 5-HIAA in the perfusates were performed using HPLC with electrochemical detection. A standard curve was generated by taking varying amounts of the compounds of interest and adding them to equal amounts of 0.9% saline. Preliminary data indicated no significant differences between standard curves established in saline and those established in perfusates collected from the LH (0.5–800 pmoles).

The HPLC system (Bioanalytical Systems, Inc., West Lafayette, IN) utilized a C-18 reverse phase column (25 cm×3.2 mm, Waters Company) coupled with a glassy carbon detector set at a potential of +0.8 V versus the reference electrode. The electronic controller was set at 5 nA/v and the recorder at 0.1 V full scale. The HPLC buffer was 0.1 M citrate and 0.1 M disodium phosphate. pH 3.5, containing 0.004% sodium octyl sulphate and 11.1% methanol by volume. The buffer was filtered and degassed under vacuum before the addition of methanol. The flow rate of the HPLC was maintained at 1.0 ml per min. Two hundred μl of each sample were injected on the HPLC without any prepurification or extraction. The compounds of interest (identified by retention times of standards) were quantified by calculating the area under the curves using an integrator (Supergrator II, Columbia Scientific Company) and their contents determined from standard curves.

RESULTS

Chromatographs of NE, DA, 5-HTP, 5-HT and 5-HIAA are shown in Fig. 1. Part A is a profile of standards. Part B is a profile of a perfusate sample collected 45 min following the SC injection of 50 mg/kg of D,L-5-HTP.

Levels of the compounds measured in the perfusate prior to and following 0.9% saline injections in two animals are shown in Table 1. Two 15 min periods were collected prior to the injection of saline indicated by an arrow, and in six 15

TABLE 1

LEVELS OF COMPOUNDS MEASURED IN THE PERFUSATE PRIOR TO AND FOLLOWING 0.9% NaCl INJECTION

			pmoles/15 min						
Compounds	Rat	Washout	0(↓)*	15	30	45	60	75	90
5-HTP	f250	1.4	1.7	1.4	2.4	2.4	2.9	2.2	2.6
	f280	3.2	2.8	2.8	1.9	1.8	1.6	1.8	1.9
5-HT	f250	0.6	0.5	0.6	0.3	0.5	0.5	0.5	0.5
	f280	0.4	0.3	0.3	0.3	0.2	0.2	0.2	0.2
5-HIAA	f250	—	4.3	2.0	2.5	1.1	1.3	0.9	1.2
	f280	0.5	0.9	0.9	0.7	0.5	0.5	0.7	1.1
DA	f250	0.8	0.7	0.7	0.6	0.5	0.6	0.6	0.7
	f280	1.7	1.4	1.9	1.7	1.4	0.8	0.8	1.4

*Arrow indicates time of 0.9% NaCl injection.

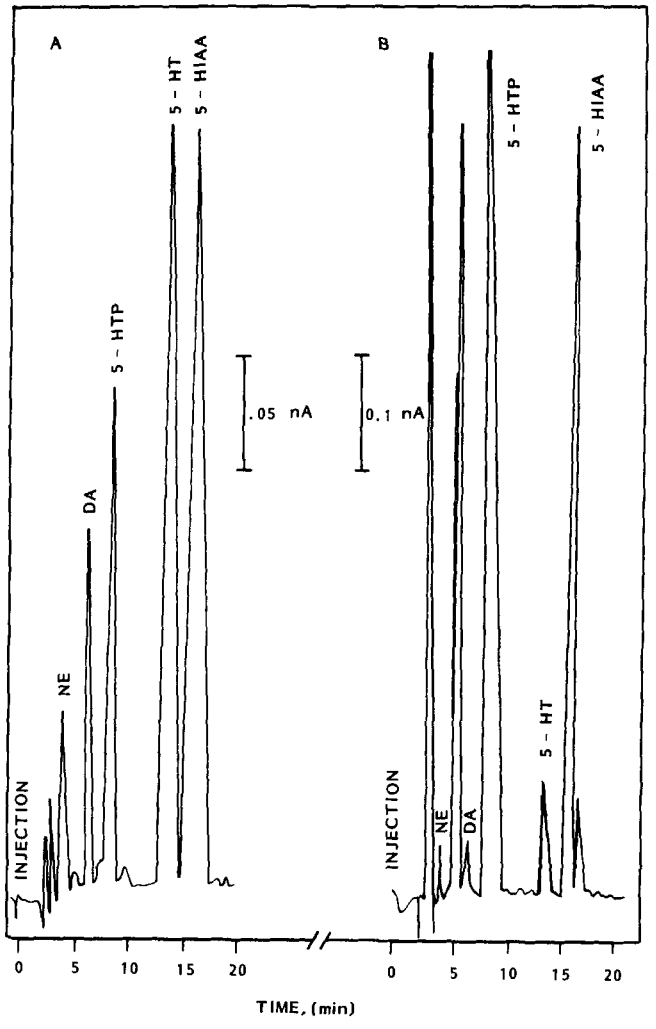

FIG. 1. Chromatographs of NE, DA, 5-HTP, 5-HT and 5-HIAA. Part A is a profile of standards. Part B is a profile of a perfusate sample collected 45 min following the SC injection of 50 mg/kg of D,L-5-HTP.

min periods following these injections. Changes in the biogenic amine levels during the period of sampling for each rat were minimal.

The number of pmol of 5-HTP, 5-HT, 5-HIAA and DA in the perfusate from rat f-250, before and after the injection of 50 mg/kg D,L-5-HTP, over a series of 15 min collection periods is shown in Fig. 2. Number of lever presses for the same time periods are shown in the lower part of the figure. Similar data are presented for rat f-252 in Fig. 3. Following peripheral injections of 5-HTP there were dramatic and immediate 5-HTP increases in brain perfusates from the LH which peaked at 15 to 30 min. In subsequent samples, 5-HTP levels began declining although they did not return to baseline values before the perfusion was terminated. Levels of 5-HT were also increased, although the increase for rat f-252 is more dramatic and immediate than for f-250. Levels of 5-HT and 5-HIAA increased following the elevation of 5-HTP in time and appeared to be leveling off, but did not return to baseline before the termination of the perfusion. Levels of DA did not change consistently or appreciably over time, during the course of the experiment. Finally, lever pressing was depressed for approximately 30 min and returned to preinjection levels by the end of the perfusion in the case of f-250 and exceeded it in the case of f-252.

Although it was possible to separate and quantify NE in perfusates, considerable difficulty was encountered with extended column use. Apparently the use of high amounts of sodium octyl sulphate (a detergent) in the buffer, effectively diminishes the capacity of the column to separate compounds near the solvent front. This, in turn, necessitates the use of even higher amounts of sodium octyl sulphate in order to improve the NE separation at the expense of the column life. The separation of the other compounds, however, is not seriously affected with 0.004% sodium octyl sulphate and the same column can be used for a considerable length of time. For this reason, data on NE are not being presented in this report.

Histological verification indicated that the cannula tips were in the LH, lateral to the fornix and medial to the cerebral penduncle.

DISCUSSION

These results indicate that the combination of push-pull perfusions and HPLC assay methods can provide a simple,

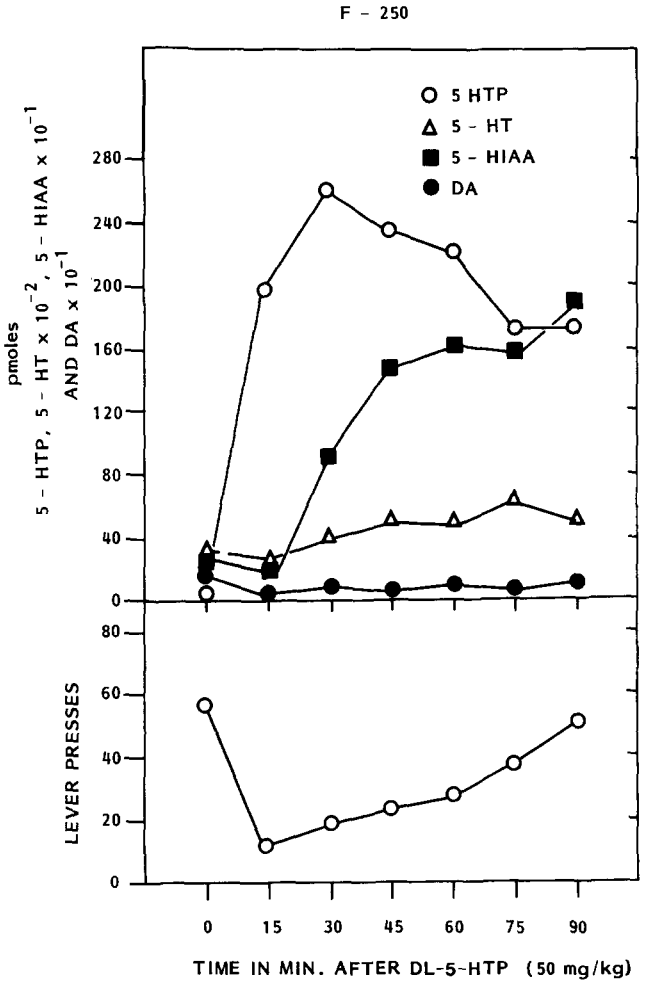

FIG. 2. Number of pmol of 5-HTP, 5-HT, 5-HIAA and DA in the perfusate from rat f-250 before and after the SC injection of 50 mg/kg, over a series of 15 min collection periods. Number of lever presses for the same time periods are shown in the lower part of the figure.

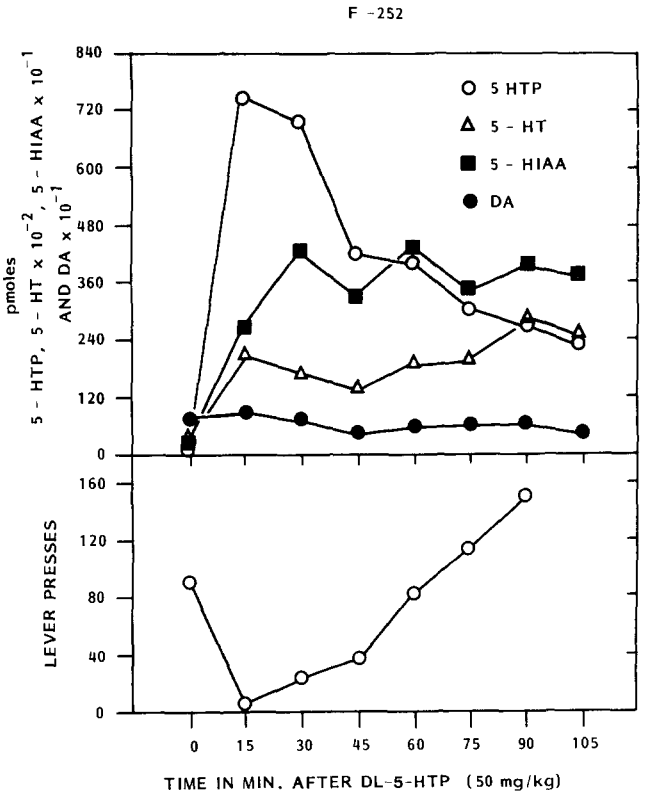

FIG. 3. Same as Fig. 2. for rat f-252.

rapid and sensitive technique for the in vivo simultaneous determination of 5-HTP, 5-HT, 5-HIAA and DA released from discrete brain areas during ongoing operant responding by a rat. The absence of any prepurification or extraction procedures after the perfusate is collected represents an additional advantage in minimizing losses due to storage. With a relatively small effort, the HPLC method described here also could be modified to measure the levels of catechol metabolites in perfusates along with the serotonergic precursor and products which we are presently able to measure. It is also noteworthy that this technique can be adapted for specific activity measurements.

The data presented suggests that these methods could be used in studying neurotransmitter changes in discrete areas of the rat brain during the period of behavioral depression following 5-HTP administration. A single dose of 50 mg/kg D,L-5-HTP resulted in time dependent increases in perfusate levels of 5-HTP, 5-HT and 5-HIAA with concomitant decreases in behavioral responding. Previous studies from our laboratories [1–7] have correlated the return to normal responding following L-tryptophan or 5-HTP administration with a return to normal in total 5-HT levels in the telencephalon. The fact that 5-HT levels in the perfusate from the LH did not return to normal levels by the time baseline responding was re-established may indicate that this area is not related to the behavioral depression seen following 5-HTP. However, it is clear that not only must more rats be studied but other areas of the brain should be perfused, including telencephalic structures. Such new data would help establish whether increases in released 5-HT and behavioral depression can be correlated, as well as elucidate other possible neurochemical-behavioral relationships.

Although the push-pull cannulation procedures and the HPLC methods have been used previously, they have never been used together with the measurement of operant responding in the freely moving rat. The results of this study demonstrate that the use of these techniques in subsequent experiments could facilitate the assessment of neurochemical changes in brain during ongoing steady-state behaviors or during the disruption of behavior following administration of drugs, precursors or other perturbations.

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