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PALEOCEREBELLAR STIMULATION INDUCES *IN VIVO* RELEASE OF ENDOGENOUSLY SYNTHESIZED [^{3}H]DOPAMINE AND [^{3}H]NOREPINEPHRINE FROM RAT CAUDAL DORSOMEDIAL NUCLEUS ACCUMBENS

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Abstract—The influence of cerebellar vermis stimulation on noradrenergic and dopaminergic activity in the nucleus accumbens was investigated in anesthetised rat. Artificial cerebral spinal fluid containing [^{3}H]tyrosine was continuously circulated through a unilateral push–pull cannula implanted in the nucleus accumbens. Fifteen-minute perfusate samples were collected serially for three consecutive 1-h periods designated pre-, during-, and post-stimulation. The stimulation was applied through a bilateral electrode located subdurally over the fifth vermal lobe. The [^{3}H]norepinephrine and [^{3}H]dopamine components in each sample were isolated by alumina extraction and high-pressure liquid chromatographic fractionation, and then quantified by liquid scintillation counting. For cannula locations in the caudal dorsomedial nucleus accumbens, levels of both [^{3}H]catecholamines were found to be significantly higher during stimulation compared to the prestimulation baselines, and [^{3}H]norepinephrine remained significantly elevated through the post-stimulation period. The relative increase during stimulation for [^{3}H]norepinephrine (130%) was nearly twice that for [^{3}H]dopamine (70%).

These results indicate that vermal activation can significantly raise both noradrenergic and dopaminergic *in vivo* activity in the caudal dorsomedial nucleus accumbens, and provide a possible mechanism for explaining previously demonstrated influences of paleocerebellum upon affective components of behavior.

Although the cerebellum was reported to influence behavioral functions nearly 40 years ago,[18] these findings were eclipsed in the following decades by extensive investigations on cerebellar control of skeletal muscle function.[10] Recent evidence for modulation of behavioral responses by the cerebellum, and particularly, the paleocerebellum (which includes the vermal cortex and the fastigial nuclei), has rekindled interest in non-motor cerebellar function. A broad range of investigations on rats, cats, and rabbits has shown fastigial influences on functions previously associated with limbic and hypothalamic stimulation or lesion.[2,5,6,8,23,25] Since neuroanatomical studies indicate that the fastigial nuclei connect to brainstem and mesencephalic catecholaminergic cell groups,[7,24,26,28] which themselves project widely to the forebrain, paleocerebellar influence on behavioral substrates is thought to occur through alteration of forebrain catecholaminergic activity. Long-term studies of forebrain levels of catecholamines (tissue homogenate assays) have indicated significant changes in these transmitters in response to a variety of cerebellar manipulations.[28,29,30] However, the application of methods for *in vivo* monitoring of short-term changes (push–pull cannula perfusion and microdialysis) under cerebellar stimulation should provide evidence for altered catecholaminergic activity more relevant to acute behavioral observations. The present investigation applied the push–pull cannulation technique of Nieoullon *et al.*[20] to determine alterations in the release of [^{3}H]norepinephrine and [^{3}H]dopamine in the nucleus accumbens under paleocerebellar stimulation in anesthetised rat. Rather than fastigial stimulation, electrical excitation of the more accessible fifth vermal lobe, which projects to the fastigial nuclei via Purkinje cell axons, was used in this study.

EXPERIMENTAL PROCEDURES

The push–pull cannulation method for *in vivo* monitoring of synaptic release in a restricted brain area, as developed by Nieoullon *et al.*,[20,21] employs continuous circulation of tritiated precursor to nerve terminals for uptake, and regular recovery and measurement of tritiated transmitters subsequently released. The following methodological description focuses only on procedures which differed substantially from the approach of those workers. The most important change was the use of high-pressure liquid chromatography (HPLC) to separate the catecholamines rather than gravity-flow columns.

Materials

L-[ring-2,3,5,6-^{3}H]Tyrosine of specific activity 95.2 Ci/mmol, L-[7-^{3}H(N)]norepinephrine, and 3,4-[7-^{3}H(N)]-dopamine were obtained from New England Nuclear (Boston, Massachusetts); norepinephrine bitartrate and dopamine hydrochloride from Sigma (St. Louis, Missouri); octyl sodium sulfate from Eastman Kodak (Rochester, New York); scintillation cocktail (Scinti Verse I) from Fisher Scientific (Pittsburgh, Pennsylvania). All other reagents were obtained from either Sigma or Fisher. The push–pull cannulas were manufactured by Plastic Products (Roanoke, Virginia).

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Abbreviations: EDTA, ethylenediaminetetra-acetate; HPLC, high-pressure liquid chromatography.

Precursor purification

Because of the very low yield of tritiated catecholamines in this type of experiment, the [^{3}H]tyrosine was purified to ensure good resolution of the [^{3}H]norepinephrine and [^{3}H]dopamine peaks during the HPLC separation step. Our simplified procedure consisted of adjusting the pH of the stock [^{3}H]tyrosine to 8.6 with 0.1 N NaOH, placing this solution in a microfilter containing 50 mg of acidified aluminum oxide, and centrifuging the filter slowly until all the fluid had passed through. The centrifugate was then cycled through this process twice more. The final product was added to artificial cerebral spinal fluid[20] to make an experimental precursor solution of about 35 μCi/ml.

Animal surgery

Fifteen Sprague–Dawley male rats (Charles River Breeders, Medford, Massachusetts), weighing 275–325 g, were used in this study. Rats were anesthetised with urethan (i.p., 1.5 g/kg) and maintained at 37°C throughout the experiment with an automatic blanket. Respiratory assistance was provided by permitting free breathing from a humidified gas stream of 95% O_2/5% CO_2 presented across the top of a T-tube inserted into the trachea. A push–pull guide cannula (0.81 mm outer diameter) was inserted into the brain stereotaxically[22] to locate its tip in the nucleus accumbens at AP 3.2 (with respect to bregma), LAT 2.0 (left side), D 6.0 (below dura). Its pedestal was then secured to the skull with dental acrylic. A flat cerebellar stimulator was inserted manually through a wide access hole in the skull 4.0 mm behind lambda. It was then slid under the dura to bring its two silver disks electrodes in firm contact with the fifth vermal lobe bilaterally (Fig. 1). Throughout the operative phase, all wound edges and pressure points were treated with Lidocaine to control pain. To minimise the effects of stress, a 90-min post-operative rest period was provided before data collection.

Run protocol

A push–pull inner cannula was inserted into the guide cannula, and the input and output ports were connected with separate flow lines to a peristaltic pump. Artificial cerebral spinal fluid was circulated by the pump at a flow rate of 0.03 ml/min from a supply reservoir, through the cannula, to a discard reservoir. Following a 30-min flow-stabilisation period, the perfusion fluid was changed to the experimental precursor solution. Under these conditions a small pocket was formed in the nucleus accumbens tissue at the cannula tip. The flow through this cavity permitted sufficient uptake of [^{3}H]tyrosine by local catecholaminergic terminals so that the release of [^{3}H]norepinephrine and [^{3}H]dopamine, subsequently synthesised by these terminals, could be detected in the cannula effluent. Special precautions were taken in establishing this flow, so that the pressure in the cavity remained at atmospheric level.

The data on each animal were obtained by collecting 12 samples from the cannula system over 3 h. Each sample consisted of 0.45 ml of cannula effluent (15-min accumulation) to which was added an equal volume of stabilising solution (10 mM thioglycolic acid, 1 mM ethylenediaminetetra-acetate (EDTA)). The first four samples monitored the spontaneous (baseline) release of both tritiated catecholamines in the nucleus accumbens for 1 h (prestimulation period). The next four monitored the release during fifth vermal lobe stimulation (stimulation period). The final four monitored the release during nucleus accumbens recovery (post-stimulation period). All samples were kept in an ice water bath during collection and then frozen at −70°C until assay. In the second hour, the stimulation consisted of biphasic square pulses, each 1.0 ms wide and 1.5 mA peak-to-peak, presented at 100 Hz. Rather than being applied continuously, the pulses were provided in 5-min trains, separated by 5-min rest periods, to discourage refraction. This paleocerebellar stimulation pattern has been previously found to provide maximal forebrain response.[15] Current spread from the stimulation site is minimal.[27] At the completion of data collection, the brain was removed for histological verification of cannula and stimulating electrode locations.

Assay procedure

Each sample was extracted on alumina, according to the method of Goldstein *et al.*,[11] to discard residual [^{3}H]tyrosine, and then separated by HPLC to isolate each of the tritiated catecholamines. The chromatograph (Bioanalytical Systems, Model LC-304) consisted of a 25 cm × 4.6 mm reverse-phase column (Biophase ODS, C-18, 5 μm spherical particles) and an electrochemical detector (set at 650 mV). The mobile phase (0.15 mM monochloroacetic acid, 2.0 mM Na EDTA, and 0.22 mM octyl sodium sulfate; adjusted to pH 3.0) eluted norepinephrine at 4–5 min and dopamine at 18–20 min when the flow rate was 2.0 ml/min. Since the quantity of either catecholamine (considering both the endogenous and tritiated forms) present in a sample was usually close to the detection limit of the HPLC, 10 ng each of authentic untritiated norepinephrine and dopamine were added to each sample before assaying in order to enhance the HPLC peaks. The fraction collector was programmed to shift tubes every minute, and, by synchronising the collection of HPLC-detector effluent following each sample injection with the appearances of the authentic catecholaminergic peaks, the tritiated components were isolated to three tubes. The contents of each of these tubes were separately added to 10 ml volumes of ScintiVerse I and counted for 10 min in a Beckman LS-230 scintillation system. The count value for each tritiated catecholamine in each sample was corrected three ways: (1) for alumina extraction losses, by monitoring assumed similar losses from the known quantities of authentic untritiated norepinephrine and dopamine added to each sample above; (2) for remaining precursor "background" ([^{3}H]tyrosine or catechols not discarded during alumina extraction), by monitoring the response to the assay procedure of pure (uncirculated) samples of precursor solution; and (3) for chromatographic separation losses and scintillation count-

Fig. 1. (a) Sagittal section of rat brain, 1.3 mm lateral to the midline, showing (approximately to scale) the locations of a push–pull cannula (PPC) in the nucleus accumbens (Nac) and a fifth vermal lobe stimulator (FVLS) on the cerebellum as employed in this investigation. The fastigial nucleus (FAST), locus coeruleus (LC), and ventral tegmental area of Tsai (VTA) may serve as intermediaries in the propagation influence upon the nucleus accumbens (see text). (Figure adapted from atlas of Pellegrino *et al.*[22]) (b) Twelve push–pull cannula tip locations within or near the nucleus accumbens (Nac) for which altered release of [^{3}H]norepinephrine and [^{3}H]dopamine under fifth vermal lobe stimulation were examined in this study. Only for the closed circle (●) positions (all located in the caudal dorsomedial aspect of the nucleus) were both tritiated catecholamines found to increase. All other locations yielded mixed release results: (◓) [^{3}H]dopamine alone rose significantly, (◒) [^{3}H]dopamine alone fell significantly, and (○) no significant change occurred in either tritiated catecholamine. For clarity the caudate putamen (CPu), anterior commissure (ac), and corpus callosum (CC) are labeled. (Figure adapted from atlas of Pellegrino *et al.*[22])

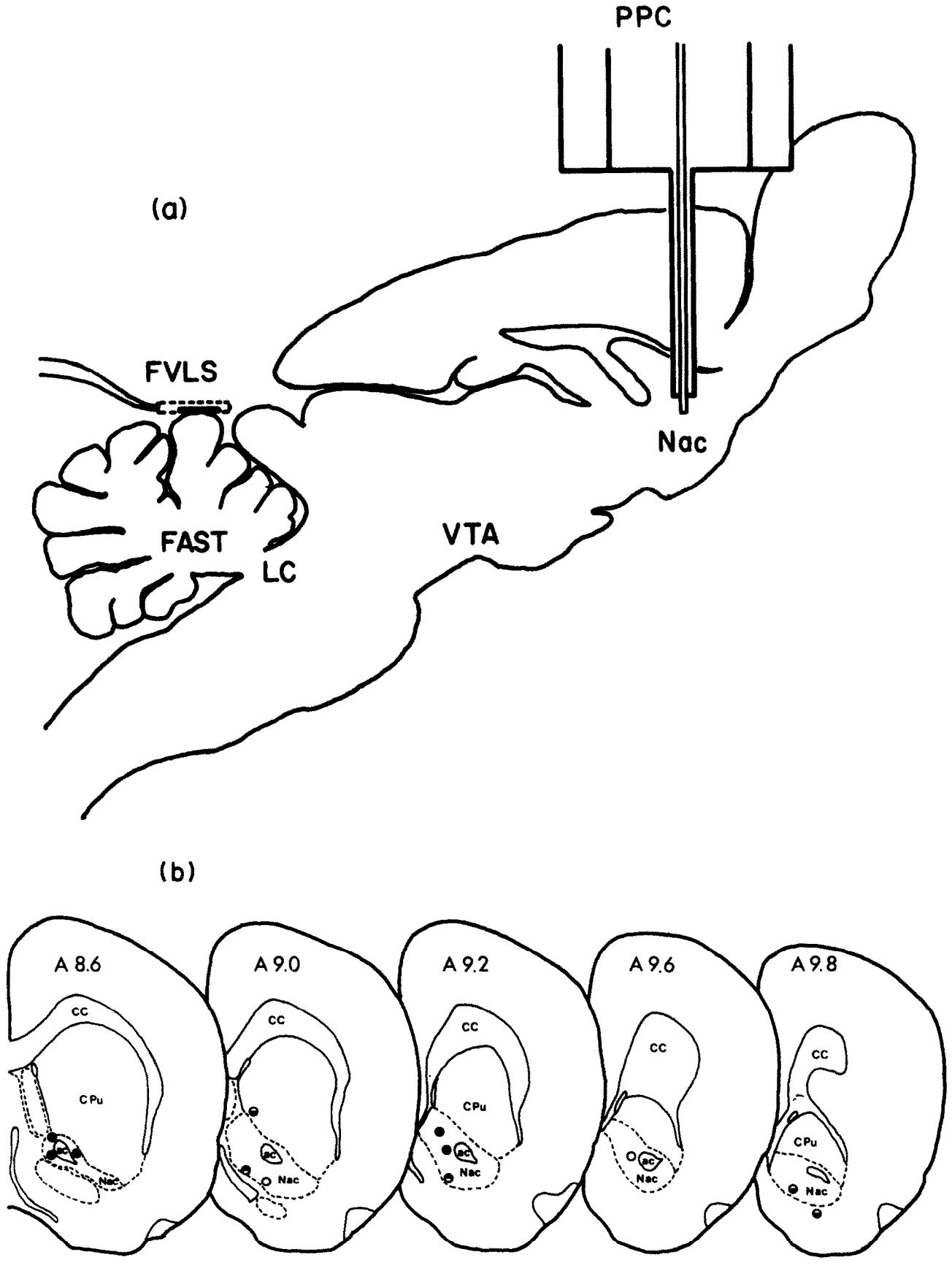

Fig. 1.

ing efficiency, by monitoring the response to the assay procedure of additional samples of pure precursor solution spiked with known quantities of authentic tritiated norepinephrine and dopamine. Alumina extraction recovery for both catecholamines averaged 64%. Precursor background levels averaged 31% of sample levels in the prestimulation period, but dropped to 14% once stimulation began. Chromatographic separation recovery averaged 67%, while scintillation counting efficiency averaged 42%.

RESULTS

Five of the rats used displayed increases in release of both [^{3}H]norepinephrine and [^{3}H]dopamine during the stimulation period compared to the prestimulation baseline. Histological examination of the brains of these animals revealed all cannulas to be located, according to the atlas of Pellegrino *et al.*,[22] in the caudal nucleus accumbens from AP +8.6 to +9.2, but only in the dorsomedial area extending from the tip of the lateral ventricle down to and surrounding the anterior commissure (Fig. 1).

Five other rats were found to have cannulas located in nucleus accumbens, but either showed no release changes or only [^{3}H]dopamine release changes. No cannula in this group was positioned in

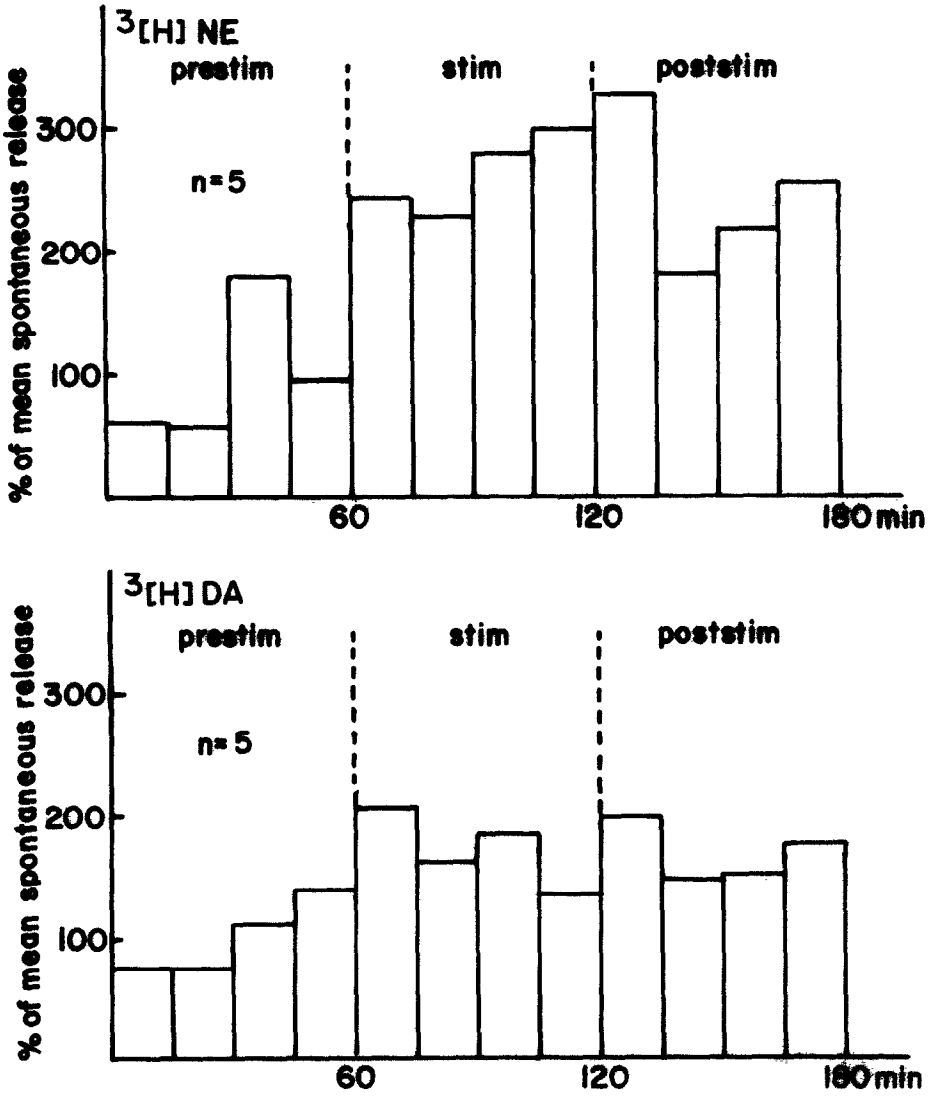

Fig. 2. Elevated release of both tritiated norepinephrine ([^{3}H]NE) and dopamine ([^{3}H]DA) in caudal dorsomedial nucleus accumbens of anesthetised rat ($n = 5$) subject to fifth vermal lobe stimulation. [^{3}H]Tyrosine was continuously circulated (by push–pull cannula) through the nucleus accumbens at a rate of 1.05 μCi/min, and the perfusate was collected in 15-min samples. The 12-sample data for each animal were first expressed as percentages referred to that animal's prestimulation average (over the first four samples) taken as 100%. The percentages corresponding to each 15-min collection period were then averaged over the animals to produce the vertical bars shown. Statistical analysis of these data in the stimulation period (see text for details) showed that releases of both [^{3}H]norepinephrine and [^{3}H]dopamine were significantly higher compared to the prestimulation period, and that the release of [^{3}H]norepinephrine was significantly higher than the release of [^{3}H]dopamine ($P < 0.05$). [^{3}H]Norepinephrine also was significantly elevated in the post-stimulation period.

the caudal dorsomedial area (Fig. 1). Two additional rats showed changes in [^{3}H]dopamine release, but had cannulas positioned just outside the borders of the nucleus accumbens (Fig. 1). The three remaining rats of the original 15 were eliminated from the study following histological examination because the stimulating electrode was not confined to the fifth vermal lobe, or the tissue cavity at the cannula tip was found to be large and irregular. The cavities in all acceptable animals were approximately spherical with diameters of about 1.1 mm, and microscopic inspection of brain slices containing cavities revealed minimal tissue degeneration.

Because of the consistent stimulation-induced elevations in release of both [^{3}H]norepinephrine and [^{3}H]dopamine observed for cannula locations in the caudal dorsomedial nucleus accumbens statistical analysis was applied to this group of animals only ($n = 5$). In combining data within this group, recognition must be given to the finding that spontaneous release values varied widely among subjects. Therefore, it was not possible to establish an experimental 3-h baseline for spontaneous release against which all animals could be compared. As originally pointed out by Nicoullon *et al.*[20] in describing this method, each animal must constitute its own control: stimulation and poststimulation values must be compared with prestimulation values for each rat. By expressing this comparison for each subject on a percentage basis (with the prestimulation average over four serial samples taken as 100% for each tritiated catecholamine), the combined results over the five animals can be displayed (Fig. 2). These percentage data were tested for significance using two-factor analysis of variance (time and tritiated transmitter) with repeated measures on both factors. The final analysis showed that the release of both tritiated catecholamines for this group of rats was significantly higher ($P < 0.05$) in the stimulation period compared to the prestimulation period. This increase continued to be significant for tritiated norepinephrine into the post-stimulation period ($P < 0.05$). [^{3}H]Dopamine remained elevated, but not significantly, in the post-stimulation period. Finally, the elevated release of [^{3}H]norepinephrine ($\sim 130\%$ increase) was significantly greater ($P < 0.05$) than the elevated release of [^{3}H]dopamine ($\sim 70\%$ increase) in the stimulation period.

DISCUSSION

We have previously reported, using the technique of *in vivo* perfusion of [^{3}H]tyrosine through specific limbic sites in cat, that fifth vermal lobe stimulation led to elevated release of undifferentiated tritiated catecholamines in the septal and hippocampal regions.[9] The present findings, using the same procedure, now establish in rat that both [^{3}H]norepinephrine and [^{3}H]dopamine are involved in catecholaminergic increases occurring in the caudal dorsomedial nucleus accumbens. These changes were significant for both tritiated catecholamines in the stimulation period, and only slowly declined in the poststimulation period, the increase for [^{3}H]norepinephrine remaining significant compared to prestimulation baseline. The tendency for the effects of long-term paleocerebellar stimulation (1 h in the present case) to be evident well beyond termination of the stimulation phase has been reported before.[9,15]

The reason for the particular response of the caudal dorsomedial area of the nucleus accumbens to the employed method is unknown. It may be that this region is especially rich in catecholaminergic terminals, or that vermal influence is stronger there. None the less, the effect was subtle even in this area. Spontaneous release averaged approximately 0.6 pg per (15 min) sample for [^{3}H]norepinephrine and 5 pg sample for [^{3}H]dopamine. Stimulated release of tritiated catecholamines at no time exceeded 10 pg per sample. Possibly other areas of the nucleus accumbens were responding to vermal stimulation, but at levels below the detection limits of the protocol used. In this connection it is interesting that the most reinforcing area for amphetamine self-administration in the nucleus accumbens is the pericommissural region,[16] which is prominent in the caudal dorsomedial aspect (Fig. 1).

In both this study and its predecessor[9] it was observed that failure to locate the cerebellar stimulator on the fifth vermal lobe invariably yielded a null response. The specificity of anterior vermis for inducing a limbic region response from cerebellar cortex stimulation has been noted previously.[15,19]

There have been several earlier attempts to measure changes in catecholaminergic activity in the forebrain under paleocerebellar lesion or stimulation.[1,21,28,29,30] However, protocols were so varied that comparisons with the present study are difficult. Albert *et al.*,[1] measuring dopamine and 3,4-dihydroxyphenylacetic acid levels in rat nucleus accumbens tissue homogenates after alpha-methyl-para-tyrosine pretreatment to inhibit dopamine synthesis, found enhanced dopamine utilisation under vermal stimulation. This is in general agreement with the present study. Snider *et al.*[28,29,30] have made extensive studies of chronic level changes and turnover of dopamine and norepinephrine in whole forebrain homogenates of rat and cat under both paleocerebellar stimulation and lesion, and conclude generally that the fastigial nuclei are excitatory to forebrain catecholaminergic mechanisms, while the vermis is inhibitory; this is clearly inconsistent with the present report.

Current knowledge of pathways connecting vermal cortex to the nucleus accumbens is too speculative to help resolve this conflict. Neuroanatomical tracing studies suggest that the fastigial nuclei project to the ventral tegmental area[24,28] and the locus coeruleus.[7,26,28] There is strong evidence that the ventral tegmental area projects to the nucleus accumbens[4]

and lesser evidence that the locus coeruleus does also.[7] A simple model of vermal influence upon nucleus accumbens can be hypothesised from these connections. Assuming that the fastigial nuclei are facilitatory to the mesencephalic/brainstem catecholaminergic cell groups, then the known inhibition of the fastigial nuclei by the Purkinje cells of the vermal cortex[10] would enable vermal stimulation to inhibit catecholaminergic activity in the nucleus accumbens. The present results deny this view, although it is acceptable to workers who examine paleocerebellar influence on larger divisions of the forebrain.[28,29,30] Since our earlier study of the influence of vermal stimulation on a variety of limbic structures supports the general occurrence of enhanced catecholaminergic activity,[9] it is unlikely that the disagreement with other workers can be explained by claiming the caudal dorsomedial nucleus accumbens represents an anomalous case.

Two other models of vermal influence may be pertinent here although they have not been pursued recently. One proposes, on the basis of evoked potential studies and neuroanatomical tracing, that the paleocerebellum connects directly to limbic nuclei.[13,14] The other model views vermal stimulation as recruiting both intra- and extra-cerebellar nuclei antidromically,[3] a concept that offers wide possibilities, but ignores the vermal lesion results. The latter proposal also de-emphasises the vermal inhibitory effect on the fastigial nuclei. It is clear that before any meaningful evaluations of these models can be made, it would be valuable to know whether direct fastigial nucleus stimulation enhances or diminishes catecholaminergic activity in the nucleus accumbens.

Paleocerebellar influence upon behavior has been suggested not only by investigations with animals,[2,25] but in experimental attempts with cerebellar stimulators to alleviate effects of motor and behavioral disorders in humans.[19] Although the success of the latter protocol remains controversial, all observers have noted an improvement in mood and behavior of these implanted patients.[12] The nucleus accumbens is a particularly useful region to monitor during procedures suspected of producing behavioral alterations. It has a high density of catecholaminergic terminals, which are considered essential for a behavioral substrate, and it is a major target of mesolimbic dopaminergic projections. It has been described as a primary center for the integration of emotionally processed information from various areas of the limbic system, and has recently been proposed as a key interface between the limbic and striatal systems for translating motivation into action.[17]

CONCLUSION

The present study, which indicates that vermal stimulation elevates catecholaminergic activity in the caudal dorsomedial aspect of the nucleus accumbens, is consistent with the view that behavioral substrates in the forebrain can be influenced by the paleocerebellum. There is a growing view that many cerebellar effects on non-motor functions may be so subtle and complex that they have gone undetected by usual observational methods.[16a] However, until reliable pathways are established from the cerebellum to forebrain limbic sites, all claimed behavioral roles for the paleocerebellum must remain tentative.

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