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EFFECTS OF NICOTINE ON THE RELEASE OF ^{3}H-NORADRENALINE FROM THE HYPOTHALAMUS

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Abstract—Nicotine injected intravenously (2 μg/kg every 30 sec for 30 min) caused an increased release of (^{3}H)noradrenaline into the effluent from the perfused third cerebral ventricle of the cat. Similar changes were observed following the administration of cigarette smoke directly into the lungs and after perfusion of the third ventricle with nicotine (2 or 5 μg/ml). An increase in the efflux of (^{3}H)noradrenaline also occurred, after perfusion with nicotine (50 μg/ml) of rat hypothalamic tissue slices. These observations are consistent with the hypothesis that nicotine causes release of noradrenaline from the diencephalon, in particular the hypothalamus.

NICOTINE can produce behavioural and physiological effects in various animal species, probably including man. Small amounts (2 μg/kg/30 sec) given intravenously to anaesthetized cats caused desynchronization of the electrocorticogram and an increased release of cortical acetylcholine.[1] Recent experiments, in the unanaesthetized cat *encéphale isolé* preparation, have shown that samples of cigarette smoke introduced into the lungs cause cortical and behavioural arousal, effects comparable with those of small amounts of nicotine injected intravenously.[2] These effects of nicotine and smoke can be considered as "stimulant" or "activating", since in many experiments the cat passed from the sleeping to the waking state. Experiments with rats, have indicated subtle effects of intravenous nicotine in modifying animal behaviour,[3] which may have a parallel in the human smoking situation.

Cholinergic mechanisms have been implicated in the EEG[1,4] and behavioural[5] effects of nicotine although there has previously been little direct evidence to support the involvement of adrenergic systems. Nicotine can act peripherally to release catecholamines from the adrenal medulla and noradrenaline from local stores in the heart and vessel walls. These factors led Burn[6] to suggest "It seems extremely likely that the pleasure of smoking is derived in part from the release of noradrenaline from its store in the brain".

Tritiated noradrenaline, injected intraventricularly in the rat, is selectively taken up by central catecholamine nerve terminals and cell bodies.[7] In the cat,[8] as in the rat,[9] intraventricularly administered (^{3}H)noradrenaline accumulates primarily in those regions lining the ventricular system which contain high levels of endogenous catecholamines. Recently, amphetamine has been shown to affect the efflux of (^{3}H)noradrenaline from cat brain when perfused through the ventricular system.[10] In several previous studies, nicotine and amphetamine have been found to possess similar actions on some measures of animal behaviour.[11] The experiments now reported were carried out

to determine the effect of nicotine injected intravenously (2 μg/kg/30 sec for 30 min) on the release of (^{3}H)noradrenaline from subcortical structures lining the third ventricle, particularly the hypothalamus. *In vitro* studies, using rat hypothalamic tissue slices confirmed that nicotine can increase the efflux of (^{3}H)noradrenaline from the diencephalon.

METHODS

In vivo

Experiments were performed on male cats weighing approximately 3 kg, anaesthetized by the intraperitoneal injection of diallyl–barbituric acid–urethane, 0·8 ml/kg. The trachea was cannulated, an open pneumothorax obtained by intubation of the thoracic cavity and artificial respiration was then applied. Two ml samples of cigarette smoke containing approximately 7 μg of nicotine (approximately 2 μg nicotine/kg for a 3 kg cat), could then be injected at 30 sec intervals directly into the airway from the respiratory pump, thus ensuring immediate passage of smoke into the lungs.[12] The femoral vein was cannulated to allow intravenous injections of nicotine, and blood pressure was monitored routinely from a femoral artery using a pressure transducer coupled to a Devices polygraph. Body temperature was maintained at 37 ± 1° with an Electrophysiological Instruments homeothermic blanket. The head was placed in a stereotaxic instrument and a stainless steel cannula inserted into the third ventricle[13] with its tip positioned ventral to the massa intermedia as shown in Fig. 2. Artificial cerebrospinal fluid (CSF)[14] was perfused through the cannula at 0·1 ml/min, the outflow from the third ventricle passing through a polythene tube inserted in the aqueduct. Having established flow, perfusion was stopped and 20 μl (20 μc) (^{3}H)noradrenaline hydrochloride (7c /mmole) were injected through the cannula into the third ventricle. In some experiments this was followed by the injection of 25 μl (5 μc) (^{14}C)inulin. The aqueductal cannula was then closed to allow uptake of (^{3}H)noradrenaline into brain tissue. Perfusion was recommenced 60 min later. After a further 90 min, when the rate of decline of radioactivity in the perfusate had slowed, 5 min samples (approximately 0·5 ml) were collected from the aqueductal cannula. Perfusion with bromophenol blue at the end of the experiment verified the cannula placement and the region of the third ventricle reached during the perfusion. Perfusate samples were diluted to 1 ml with CSF, and 100 μl of the resulting solution added to 15 ml of a dioxan–ethanol–xylene based scintillator.[15] Radioactivity in the samples was measured on a Model 3375 Packard Liquid Scintillation Spectrometer to an accuracy of 1 per cent. In some experiments, the proportion of radioactivity represented by (^{3}H)noradrenaline and (^{3}H)normetanephrine was estimated by the method of Carr and Moore.[10]

The total amount of radioactivity appearing in the perfusion fluid varied between experiments. To overcome this variation and to provide an acceptable method for demonstrating changes in output, a proportional measure for radioactivity was adopted. The immediate pre-drug sample was regarded as unity and radioactivity appearing in the perfusate before and after that sample was expressed proportionately. Using this approach, it was possible to average results for control experiments, and for those in which intraventricular or intravenous nicotine evoked changes in the output of radioactivity.

In vitro

Experiments were carried out using slices of rat hypothalamus. The animals were killed by cervical dislocation, the brain removed and the hypothalamus isolated.[9] The tissue was washed in physiological saline, blotted and chopped into segments 0·75 mm square using a McIlwain tissue chopper. The slices were incubated with 10^{-7} M (^{3}H)noradrenaline (100 μc) in oxygenated Krebs–Ringer-bicarbonate solution at 37° for 30 min. In some experiments 10^{-7} M (^{14}C)leucine (5 μc) was added to the incubation medium. Following incubation, the slices were removed by Pasteur pipette and placed in the well of the perfusion vessel (Millipore Filter Holder SX0001300).[16] To contain the tissue slices a disc of Whatman No. 1 filter paper was placed in the filter holder. The slices were perfused with oxygenated Krebs–Ringer solution usually at a rate of 0·6 ml/min. However, slower perfusion rates were sometimes employed. Three ml perfusate samples were collected in a fraction collector. The first six fractions were discarded owing to the steep rate of decline in radioactivity. After that time, eighteen fractions were collected, six before, during and following drug administration. For studying drug effects, nicotine was added to the perfusion solution in the required concentration and the pH adjusted to that of Krebs–Ringer solution. Total activity in 500 μl portions of the fractions were counted directly. To separate (^{3}H)noradrenaline and (^{3}H)normetanephrine for quantitative assay a further 100 μl of each perfusate sample was submitted to ascending paper chromatography on acid washed Whatman No. 3MM paper using a phenol–hydrochloric acid system. Noradrenaline and normetanephrine added to the samples as markers were identified by spraying respectively with alkaline ferricyanide and ninhydrin. Following identification, the spots were isolated and cut into two or three segments, each segment being added to 15 ml scintillator containing 1 ml water and 4 per cent Cab–o–Sil (Packard Instruments Ltd.). Each vial was then shaken for 15 min on a BTL Vortex Shaker which resulted in disintegration of the paper. Radioactivity in the samples was measured as described previously.

Drugs used were as follows: (carboxy-^{14}C)inulin, L-(U-^{14}C)leucine, DL-(7-^{3}H)-noradrenaline (The Radiochemical Centre, Amersham), DL-(7-^{3}H)normetanephrine (New England Nuclear Corporation, U.S.A.), (-)-(^{14}C)nicotine hydrogen tartrate (synthesized by Dr. H. Roderick at these laboratories) and nicotine hydrogen tartrate (B. D. H.). Doses refer to the base.

RESULTS

In vivo

Nicotine injected intravenously (2 μg/kg every 30 sec for 30 min) or perfused through the third ventricle (5 μg/ml for 30 min) increased the efflux of (^{3}H)noradrenaline into the perfusate as illustrated in Fig. 1. In six out of sixteen experiments in which nicotine was injected intravenously there occurred an increased efflux of (^{3}H)-noradrenaline from the third ventricle which was significantly different ($p < 0·01$) from the (^{3}H)noradrenaline appearing in the control samples. The maximum output occurred 25 min after the start of nicotine injections. In the remaining experiments, slight changes in the output of radioactivity were also observed following or during the intravenous administration of nicotine, but were not significantly different from the controls. When perfused through the third ventricle in a concentration of 5 μg/ml for

30 min, a significant increase ($p < 0.05$) in the output of (^{3}H)noradrenaline into the perfusate samples occurred in six out of ten experiments. The mean responses are also shown in Fig. 1. The total amount of nicotine administered during drug perfusion was 15 μg. The maximum response, which was greater than that observed following intravenous administration, was apparent 20 min after commencing the perfusion with nicotine. The onset of drug effect also occurred somewhat earlier than for intravenous nicotine. Smaller amounts of nicotine (2 μg/ml for 30 min) were also effective in evoking the release of radioactivity in five out of twelve experiments. However, the mean effect was smaller than that observed with 5 μg/ml.

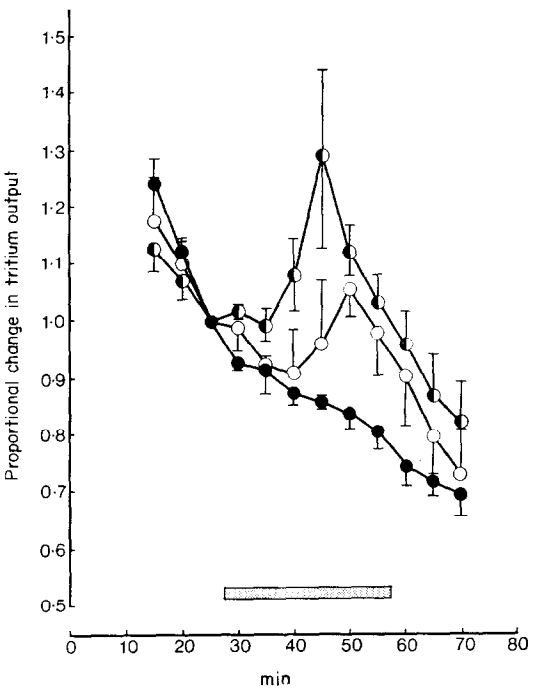

FIG. 1. The proportional release of [^{3}H] noradrenaline from the third ventricle of the anaesthetized cat by intravenous nicotine (2 μg/kg every 30 sec for 30 min), (○); intraventricular nicotine (5 μg/ml), (◐); and saline control (●). Shaded bar represents the period during which drug or saline was administered. Each graph shows the mean ±S.E. for six experiments.

To determine the specificity of the release of (^{3}H)noradrenaline following ventricular perfusion of nicotine, (^{14}C)inulin and (^{3}H)noradrenaline were injected simultaneously in some experiments. In those experiments in which the greater concentration of nicotine (5 μg/ml for 30 min) was perfused through the third ventricle, there occurred a small increase in the output of (^{14}C)inulin which paralleled that of (^{3}H)noradrenaline.

In some experiments, the amount of total radioactivity represented by both (^{3}H)-noradrenaline and (^{3}H)normetanephrine in the perfusate samples was measured. Figure 2 illustrates two experiments in which effects of intravenous nicotine (2 μg/kg every 30 sec for 30 min) were compared with a control experiment where only saline was administered. In the experiment illustrated in Fig. 2A nicotine caused an almost immediate rise in the concentration of (^{3}H)noradrenaline which was maximal 25 min

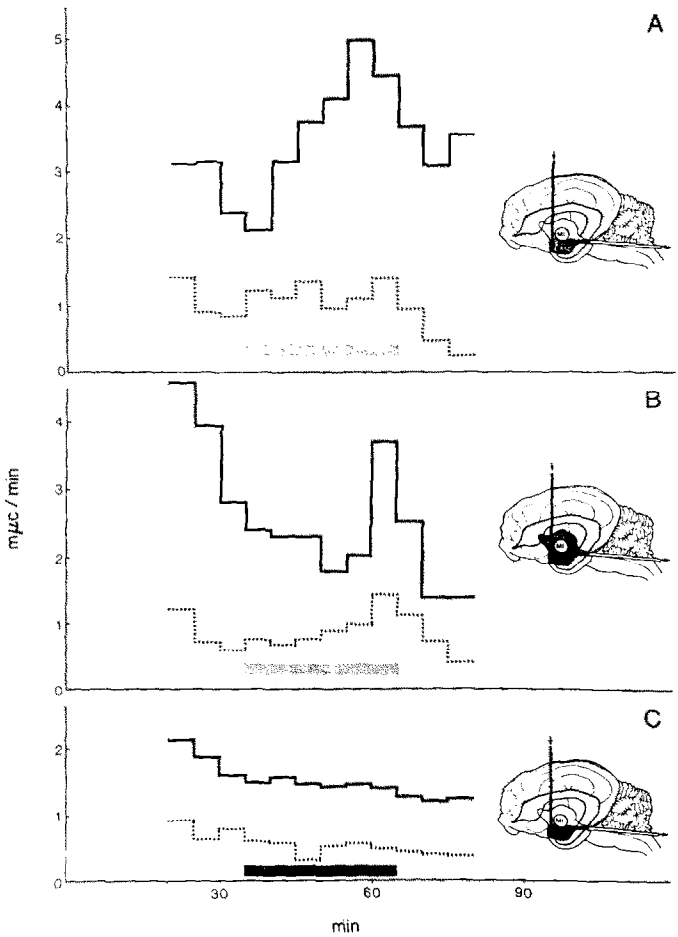

FIG. 2. The release of [^{3}H]noradrenaline (——) and [^{3}H]normetanephrine (– – –) from the third ventricle of the anaesthetized cat before, during and after the intravenous injection of nicotine, 2 μg/kg (shaded bar, A and B) or saline (solid bar, C) every 30 sec for 30 min. Inserts: Ventricular system of the brain showing position of inflow and outflow cannulae, and the region (shaded area) of the third ventricle perfused in each experiment. MI—*massa intermedia*.

after the start of drug administration. In Fig. 2B the onset of drug effect was somewhat delayed, the maximum response in this experiment occurring after 30 min. The magnitude and time of onset of the increased release varied between experiments. In the experiments of Fig. 2 radioactivity due to (^{3}H)noradrenaline, expressed as a percentage of the total radioactivity of the perfusate sample, rose from "pre-drug levels" of 32–38 per cent to peak values of 45–52 per cent during nicotine administration. These peak values for (^{3}H)noradrenaline coincided with the peak values for total radioactivity. Although a similar trend was apparent for (^{3}H)normetanephrine the changes did not always occur in parallel. Careful post-mortem dissection of the brain following perfusion with bromophenol blue, revealed staining of different regions of the third ventricle, thus indicating the specific diencephalic structures which had been in contact

with the perfusion fluid. In the experiments of Fig. 2A and 2C perfusion had been restricted to the ventral half of the third ventricle, whereas in the experiment illustrated in Fig. 2B perfusion included both dorsal and ventral aspects.

Changes in volume of the perfusate samples ranging from 0·48–0·54 mls occurred during the experiments. There were no correlations with changes in output of radioactivity and any increase in volume was never sufficient to account for the increased output of (^{3}H)noradrenaline following nicotine administration.

In three out of five experiments, 2 ml samples of cigarette smoke introduced into the lungs every 30 sec for 30 min caused an increase in the efflux of (^{3}H)noradrenaline as shown in Fig. 3. The radioactivity in the perfusate samples was again expressed as a

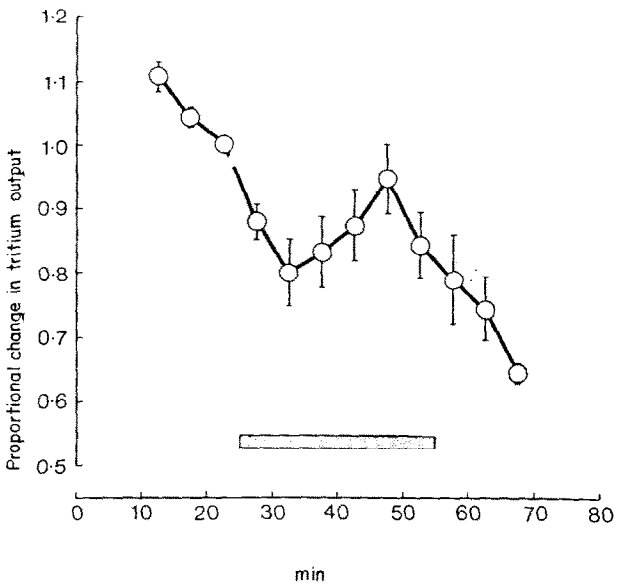

FIG. 3. The proportional release of [^{3}H]noradrenaline from the third ventricle of the anaesthetized cat by cigarette smoke introduced directly into the lungs (2 ml samples every 30 sec for 30 min). Shaded bar represents the period of smoke administration. Graph shows the mean ±S.E. for three experiments.

mean of the proportionate values for individual experiments. A maximum response was apparent 25 min after commencing smoking, the effect thus resembling that of intravenous nicotine.

In vitro

The effect of nicotine on the output of (^{3}H)noradrenaline from perfused rat hypothalamic slices is shown in Fig. 4 and is compared with control experiments. At a flow rate of 0·6 ml/min, 1 mM nicotine caused a significant increase in the output of radioactivity into the perfusate, the peak occurring after 10 min. Output of radioactivity in all the *in vitro* experiments was very reproducible and thus the absolute activities could be summed for each series of experiments. Under these conditions no effect on (^{3}H)-

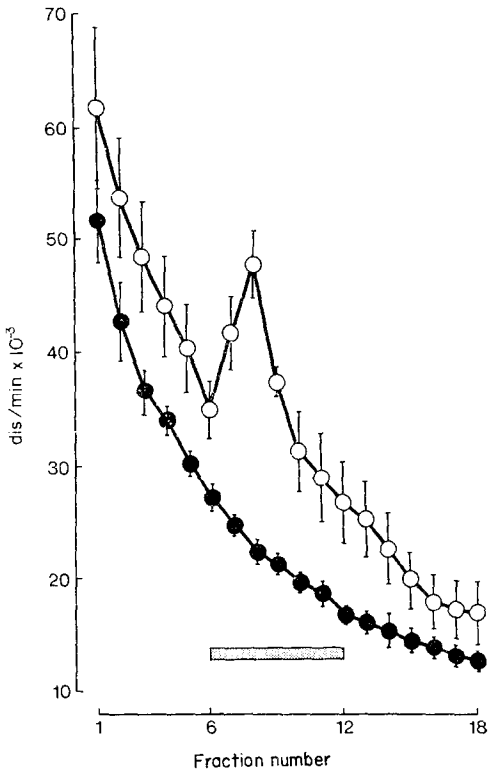

FIG. 4. The release of [^{3}H]noradrenaline from rat hypothalamic slices by nicotine (1 mM), (○); and saline control (●). The shaded bar represents the period during which drug or saline was administered. Each graph shows the mean ±S.E. for three experiments.

noradrenaline output could be demonstrated with 0·3 mM nicotine, whereas 0·6 mM reduced the rate of decline of the decay curve.

Fractionation of the radioactivity demonstrated that (^{3}H)noradrenaline comprised approximately 50 per cent of the total radioactivity of the pre-drug samples rising to 60–65 per cent during drug administration. As with the *in vivo* experiments the peak (^{3}H)noradrenaline level coincided with that of the total radioactivity. (^{3}H)normetanephrine comprised some 15 per cent of the total activity and showed similar responses to (^{3}H)noradrenaline. That nicotine was releasing (^{3}H)noradrenaline by a specific effect was demonstrated by double label experiments as illustrated in Fig. 5. The tissue slices were preincubated with both (^{3}H)noradrenaline and (^{14}C)leucine and whereas 5 mM nicotine caused a rise in the output of noradrenaline no change in leucine output was observed.

Nicotine (5 mM) and potassium (40 mM) caused an increased release of (^{3}H)-noradrenaline from the hypothalamic tissue slices when tested separately although the response was not maintained by continuous perfusion with either drug. Nicotine, however, was still effective in causing an increased release of (^{3}H)noradrenaline immediately following perfusion with potassium.

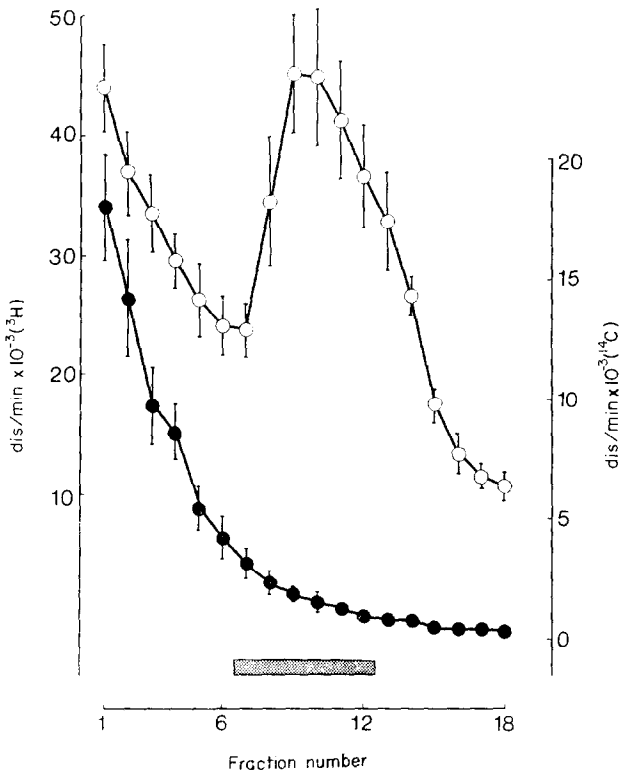

FIG. 5. The release of [^{3}H]noradrenaline, (○); and [^{14}C]leucine, (●) from rat hypothalamic slices by nicotine (5 mM). The tissue slices were pre-incubated with the labelled compounds prior to perfusion. The shaded bar represents the period during which nicotine was administered. Each graph shows the mean ±S.E. for three experiments.

Lowering the perfusion rate increased the sensitivity of the tissue slices to nicotine and at 0·2 ml/min a significant increase in output of (^{3}H)noradrenaline was produced by 0·3 mM nicotine, whereas this concentration of nicotine was ineffective at a flow rate of 0·6 ml/min.

DISCUSSION

Numerous investigators have demonstrated the occurrence of noradrenaline in the central nervous system, where it may have a functional role as a neurotransmitter. Although occurring in many brain regions, noradrenaline appears in relatively high concentrations in the hypothalamus of various species including cat,[17] dog[17] and man.[18] Carr and Moore[8] have recently shown that after injection into the third ventricle of the cat, (^{3}H)noradrenaline is concentrated in the hypothalamus and to a lesser extent in areas caudal to the third ventricle. In the present experiments, in which perfusion was restricted to the third ventricle, the increased release of (^{3}H)noradrenaline caused by intravenous or intraventricular nicotine could have occurred only from subcortical structures forming the walls of that ventricle. Furthermore, in experiments in which dye studies indicated that perfusion had been restricted to an area of the third

ventricle lying ventral to the *massa intermedia*, the increased release of (^{3}H)noradrenaline occurred most probably from the hypothalamus which forms the floor and lateral walls of that region. However, the release of (^{3}H)noradrenaline also from structures forming the rostral end of the aqueduct cannot entirely be excluded, although only relatively small amounts of (^{3}H)noradrenaline are concentrated in this region following injection into the third ventricle.[8] Any contribution from this source to the overall output of (^{3}H)noradrenaline in the experiments now reported is therefore likely to be minimal.

Intraventricular nicotine (5 μg/ml) evoked a more frequent and greater release of (^{3}H)noradrenaline than intravenous nicotine. This finding is consistent with the results of previous studies which have shown that the concentration of nicotine in the hypothalamus, after intravenous injection, approaches 200 ng/gm tissue,[19] whereas after intraventricular administration the tissue level reaches 800 ng/gm. The effect of smoke was almost identical to that of intravenous nicotine with regard to both magnitude of response and time of onset.

The finding that intraventricular nicotine increased the efflux of the extracellular marker (^{14}C)inulin in parallel with (^{3}H)noradrenaline, indicated that nicotine may be modifying extracellular and not necessarily intracellular stores of noradrenaline. In contrast to the observations of Carr and Moore[20] it was found that amphetamine (100 μg/ml) likewise caused an increased release of (^{14}C)inulin into the perfusion fluid. However, in the *in vitro* situation using rat hypothalamic tissue slices, nicotine caused a reproducible increase in the output of (^{3}H)noradrenaline from the tissue without affecting the release of the amino acid marker (^{14}C) leucine. This suggests that nicotine causes release of noradrenaline from within the cell by a specific mechanism rather than by a more general action on cell permeability. That nicotine evoked the release of (^{3}H)noradrenaline *in vitro* when potassium was no longer effective suggests differences in the mechanism of action.

Although the levels of nicotine required to elicit a response *in vitro* were relatively high it is possible to relate the results to those of the *in vivo* studies in the cat. Reducing the flow rate increased the sensitivity of the rat hypothalamic tissue slices to nicotine, 0·3 mM eliciting a reproducible release of (^{3}H)noradrenaline at a flow rate of 0·2 ml/min. Higher drug levels are frequently required in *in vitro* studies to evoke transmitter release and species may also be important with regard to difference in sensitivity. Therefore it seems probable that in the *in vivo* studies some contribution to the overall output of (^{3}H)noradrenaline could occur from within the cell although this may be masked by effects of nicotine on the extracellular fluid spaces. Since it is known that intravenous nicotine can increase cerebrospinal fluid pressure[21] and cerebral blood flow* by an action on the cerebral vasculature this could produce a "squeezing" of the extracellular fluid compartments with a resultant increase of the constituents into the perfusion fluid.

It has previously been demonstrated that chronic nicotine treatment causes an increase in the rate of disappearance of intraventricular (^{3}H)noradrenaline from rat brain inferring an increase in the synthesis and utilization of noradrenaline.[22] An increased turnover of whole brain catecholamines occurred in rats exposed repeatedly to cigarette smoke[23] and a decrease in noradrenaline content of rat diencephalon was observed following a single intraperitoneal injection of nicotine.[24] These observations are consistent with the present findings.

The intraventricular administration of noradrenaline facilitates the effects of electrical stimuli on specific neural pathways within the brain.[25] In further experiments noradrenaline was released at central synapses by electrical stimulation and by amphetamine suggesting that the release of this neurotransmitter from structures within the brain was responsible at least in part for changes in behaviour.[26] Nicotine and amphetamine have similar actions on varying types of behaviour in rats.[11] Likewise, nicotine resembles amphetamine in its ability to facilitate the effects of electrical stimuli in rats with chronically implanted hypothalamic electrodes.[27] That nicotine has now been shown to release noradrenaline from cat and rat hypothalamus suggests that one of its actions may be to modify synaptic transmission in an adrenergic system. In support of the present findings that nicotine can act in some regions of the brain by modifying the release of noradrenaline is the recent demonstration that nicotine mimics the action of noradrenaline at certain sites within the monkey hypothalamus.[28]

Finally, it is known that the intraventricular injection of noradrenaline can inhibit the stress induced release of adrenocorticotrophic hormone in the dog.[29] A further possibility thus exists regarding the action of nicotine on the central nervous system. If a similar mechanism functions in other species, particularly in man, nicotine could alleviate the effects of stress by the central release of noradrenaline.

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