the Indus River; the two larger skeletons are located at the California Academy of Sciences (CAS 14921 and 14922), and the smallest is at the Museum of Comparative Zoology (MCZ 52306).

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Intracranial Drug Implants: An Autoradiographic Analysis of Diffusion

Abstract. *Labeled crystalline atropine, administered to the hypothalamus of rats, remained strictly localized in a sphere, 1.0 to 1.8 millimeters in diameter, during the first 3 minutes. A similar distribution obtained after 1 hour. At intermediate times, slightly elevated radioactivity, reflecting concentrations 2,000 to 10,000 times below behaviorally effective doses, was observed several millimeters from the implantation site.*

Local changes in the concentration of neurohumors and related substances have been used to selectively activate or inactivate functionally defined pathways in areas where the proximity of different neural systems has limited the effectiveness of traditional techniques (*1*). The usefulness of drug-injection procedures depends on the degree of localization achieved. MacLean demonstrated (*2*) that the injection of even small quantities of fluid under pressure produces fairly extensive diffusion. We have attempted to circumvent the problems inherent in this technique by permitting the drug to go into solution in the brain, using the extracellular fluid as the solvent (*3*). This approach has its own problems in that dosage parameters are difficult to estimate, but our behavioral and electrophysiological data consistently show that diffusion is limited to a sphere about 1.0 mm in diameter. Dye-diffusion and autoradiographic studies have confirmed this estimate (*4*). Autoradiography, however, is limited by spread of radioactivity during histological processing and film mounting. This type of potential artifact is obviated in the dry-mount procedure with unfixed and unembedded freeze-dried sections (*5*), as applied in our experiment.

It is possible to obtain a map of positive and negative injection sites and thus to establish directly the extent of effective diffusion. However, the ubiquity of positive placements in some regions of the brain makes such an approach difficult for some drugs, and it has been suggested (*6*) that a ventricular distribution of these drugs may account for at least some of their effects. Such an interpretation may apply to the observation that an implant of atropine at any one of several carbachol-sensitive sites blocks the drinking which is normally elicited by the administration of carbachol (carbamylcholine chloride) to sites in the hypothalamus, preoptic area, septal area, and hippocampus (*7*). Contralateral as well as ipsilateral interacting sites have been described, and it is hard to account for the interaction in terms of an atropine blockade of direct neural projections to the carbachol implantation site.

Since the ventricular distribution hypothesis limits the usefulness of all procedures for injection of drugs into the central nervous system, we decided to use an autoradiographic method which makes it possible to limit spread of radioactivity after the tissue is dead (*5*). We studied the pattern of atropine diffusion under conditions which produce a blockade of the behavioral effects of intracranial carbachol.

In the first experiment, cannulas were implanted stereotaxically into the lateral hypothalamus of ten rats. One week after surgery, the placements were shown to be sensitive to carbachol, since the administration of 0.5 to 5.0 μg of carbachol to the lateral hypothalamus elicited an intake of at least 10 ml of water. At least 24 hours later, 1.0 to 5.0 μg of ^{3}H-labeled atropine (*8*) was administered to the same site. The animals were decapitated 2 to 16 minutes later (the behavioral effects of centrally applied atropine typically appear within 1 to 2 minutes).

In subsequent experiments, cannulas were implanted stereotaxically into the medial septal area as well as into the lateral hypothalamus of 14 rats. Both placements were shown to be carbachol-sensitive by the elicitation of a water intake of at least 10 ml within 30 minutes after the application of 0.5 to 5.0 μg of carbachol. At least 24 hours later, 0.5 to 5.0 μg of carbachol was again applied to the septal area of all rats. Thirty seconds after the animals began to drink, 1.0 to 5.0 μg of labeled atropine was placed into the lateral hypothalamus. The animals were then returned to the test situation and permitted to drink. Water intake ceased within 1 to 4 minutes after the implantation of atropine. The animals were decapitated at least 1 minute after the water intake ceased (dropped to zero). To obtain a representative sample of the drug diffusion, animals were decapitated 2, 3, 5, 6, 7, 8, 9, 12, and 60 minutes after the application of ^{3}H-atropine. After the animals were decapitated, the area surrounding the tips of both cannulas was excised while the brain was kept on a petri dish cooled on ice. Within 5 minutes after the decapitation, areas of the brain were frozen by immersion in liquid nitrogen. Drug diffusion may have continued between decapitation and freezing. The frozen tissue was used for (i) the preparation of 2-μm freeze-dried sections for dry-mount autoradiography or (ii) liquid-scintillation counting of radioactivity in blocks of tissue weighing approximately 10 mg which encompassed the site of atropine implantation. The radioactivity of comparable blood samples, taken at the time of decapitation, was also determined by liquid-scintillation counting. The autoradiograms were evaluated by counting silver grains and by obtaining densitometric estimates at exposure times from 2 weeks to 2 months.

The results (Fig. 1) are remarkably consistent, in view of the wide range of drug action and exposure times, in showing that the application of labeled atropine in crystalline form produced a pattern of intense radioactivity which remained restricted, even after 1 hour, to a sphere of about 1.0 to 1.8 mm in diameter. There was, in many cases,

a tendency for the radioactivity to appear in a pear-shaped area, extending some distance up the shaft of the implant; this may be an artifact due to the removal of the cannula at the time of decapitation. At short (2 minutes) or long (1 hour) intervals of drug action, there was no detectable activity outside this sphere of intense radioactivity. At intermediate times of drug action, notably from 6 to 12 minutes, slightly elevated counts of silver grains were found as far from the implant site as the contralateral hypothalamus or medial thalamus. An estimate (*9*) of the quantity of drug which is represented by these elevated silver grain counts suggests that all concentrations outside the sphere of intense radioactivity were approximately 2,000 to 10,000 times lower than those needed to produce overt effects (that is, a blockade of the carbachol-induced drinking). Microscopic analysis of the distribution of silver grains showed a preferential concentration over neuropil.

An analysis of the implantation site in the septal area where carbachol was presumably acting at the time of the atropine-induced blockade of its action failed to show any counts of silver grains above the background amount. Analysis of the ventricular spaces nearest the site of atropine administration failed to show concentrations of radioactivity.

The principal route of transport away from the injection site appeared to be the vascular system. Autoradiograms prepared between 5 and 12 minutes after the implantation of atropine showed silver grain concentrations in blood vessels as depicted in Fig. 1E (inset). Scintillation counting revealed only small amounts of radioactivity in the general circulation at all intermediate drug-action times. At 2 minutes and at 1 hour the blood count was zero. A comparison of the scintillation-counting data obtained from tissue samples surrounding the atropine implant with those obtained from blood samples shows that the concentration of atropine in the general circulation did not, in all probability, affect cholinergic transmission

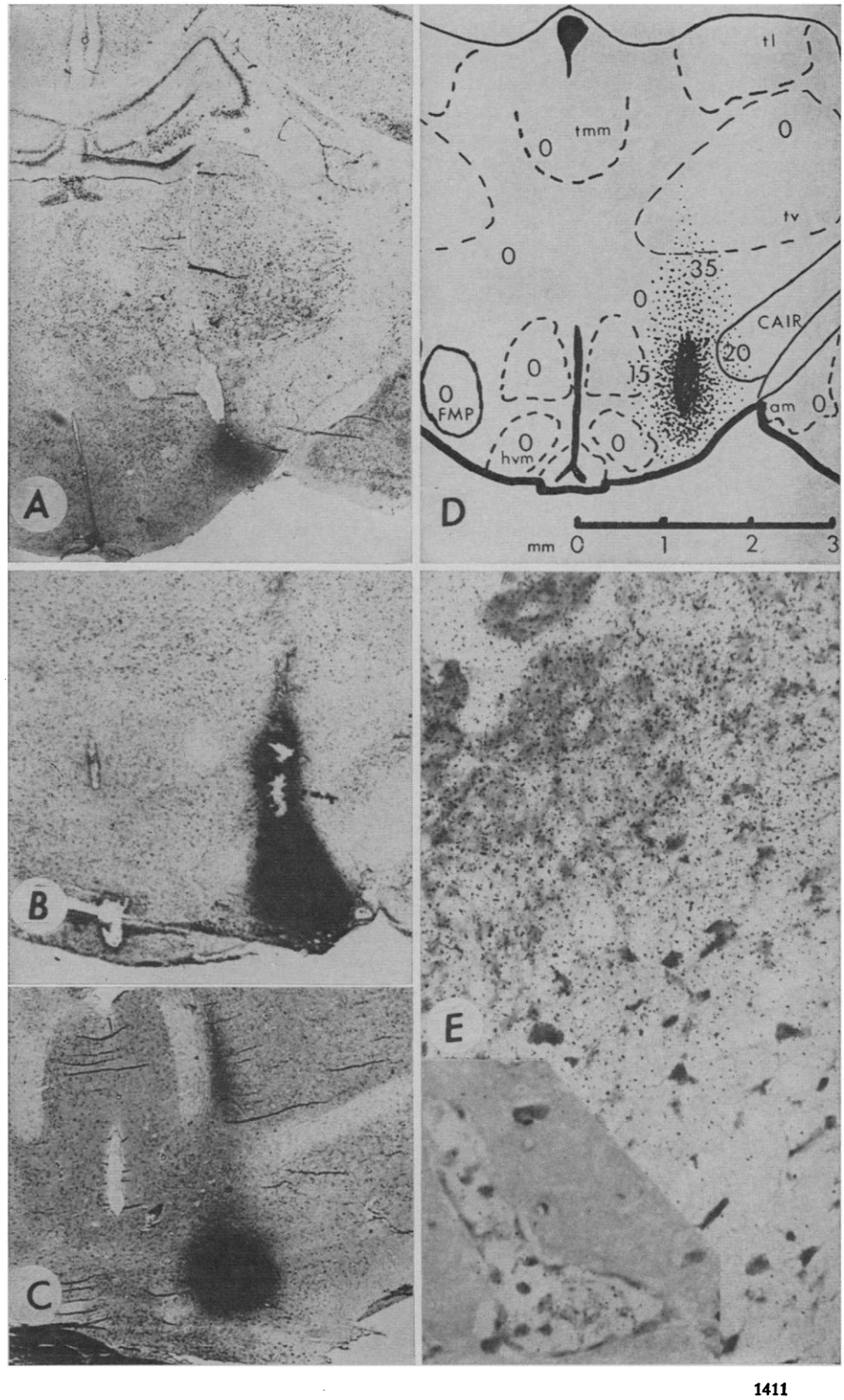

Fig. 1. Dry-mount autoradiograms of frontal sections from rat brain showing the site of ^{3}H-atropine implantation in the centrolateral (A) or posterolateral (B and C) hypothalamus. The black rounded areas indicate the spread of intense radioactivity. One hour (A) after the implantation, radioactivity is still visible at the implantation site, although the diameter appears smaller than at 12 minutes (B) and at 5 minutes (C). Part D shows in a schematic drawing the results of silver grain counting (the numbers represent silver grain counts per 250 by 250 μm area) from autoradiograms obtained 2 minutes after implantation, when carbachol-induced drinking was first blocked. Two minutes after implantation, no radioactivity could be discovered in tissue structures more than 1 mm from the core of the implantation site, even after 2 months' exposure time of the autoradiograms. *am*, Nucleus amygdaloideus medialis; *CAIR*, capsula interna, pars retrolenticularis; *FMP*, fasciculus medialis prosencephali; *hvm*, nucleus ventromedialis hypothalami; *tl*, nucleus lateralis thalami; *tmm*, nucleus medialis thalami, pars medialis; *tv*, nucleus ventralis thalami. Part E is a dry-mount autoradiogram at a higher magnification (× 100) from a frontal section of the implantation area, obtained 2 minutes after ^{3}H-atropine implantation, exposure time 14 days. The inset at the bottom of part E demonstrates transport of radioactive material in a blood vessel, 24 days' exposure time, × 200. All sections were 2 μm thick and were stained with methylgreen-pyronin.

at distant sites. Near the implantation site of threshold doses of atropine, 66,000 to 88,000 dpm (disintegrations per minute) per milligram of tissue (dry weight) were recorded as compared to 1 to 6 dpm/mg of blood (dry weight) from the general circulation.

Small quantities of atropine, applied in crystalline form (*3*), seem to produce an effective spread which appears to be limited to a sphere of 1.0 to 1.8 mm in diameter. The quantity of the drug, within the limits of our dosage, appeared to increase the intensity of the activity within this sphere but not to significantly increase its diameter. Electrophysiological observations (*10*) confirm this finding. Microelectrode recordings obtained from cells more than approximately 1.5 mm from the tip of the drug implant do not, typically, react to even fairly large doses of the drug. We emphasize that our observations apply only to small quantities of atropine and perhaps closely related chemical substances which are permitted to dissolve in the tissue fluids, by a technique which does not induce mechanical forces due to the injection of liquids under pressure.

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8. ^{3}H-Atropine, specific activity 434 mc/mmole, was generally labeled by an exchange procedure and purified by sublimation and crystallization. Its radiochemical purity was determined by thin-layer chromatography (Amersham/Searle, Des Plaines, Illinois). The chemical nature of the radioactive material at the time of tissue excision was not determined.
9. Silver grain counts in areas remote from the implantation site gave up to 50 to 100 silver grains per 625,000 μm^2 in autoradiograms obtained between 5 and 12 minutes after the implantation. Considering that the medium range of β-particles from tritium is 1 μm and about 20 disintegrations statistically result in 1 silver grain [A. W. Rogers, *Techniques of Autoradiography* (Elsevier, Amsterdam, 1967)], it was calculated that 1 mg (dry weight) of brain contained 4.9×10^{-6} μc of radioactivity for 50 randomly distributed silver grains. This is equivalent to 3.3×10^{-6} μg of atropine. The radioactivity at the implantation site proper ranged from 0.02 to 0.03 μg of ^{3}H-atropine per milligram (dry weight), calculated from 10- to 12-mg samples of tissue containing the implantation core.
10. E. Kent and S. P. Grossman, unpublished observations.
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Hypobaric Hypoxia: Effects on Early Development of Tryptophan Oxygenase in Neonatal Rats

Abstract. *Despite a reduction in liver and body weights of neonatal rats born and reared at a simulated altitude of 5790 meters (oxygen pressure, 76.36 millimeters of mercury), the hepatic enzyme tryptophan oxygenase develops prematurely in these stressed animals as compared to controls reared at sea level. Also, the specific activities remain distinctly elevated through the first 9 days of age; thus, the competence for premature synthesis of tryptophan oxygenase is confirmed in neonatal rats.*

Premature development of enzymes has been effected by the administration of chemical stimuli either directly to the fetus or by way of the maternal circulation. Intraperitoneal injection of thyroxin into fetal rats increases the activities of liver glucose-6-phosphatase and reduced nicotinamide-adenine dinucleotide phosphate : cytochrome c oxidoreductase (*1*). Similarly, the fetal intraperitoneal injection of dibutyryl cyclic adenosine monophosphate (AMP) causes the premature induction of both tyrosine aminotransferase and glucose-6-phosphatase (*2*); injections *in utero* of glucagon into fetal rats cause an increase in tyrosine aminotransferase activity (*3*). L-Tyrosine fed to pregnant rabbits increased significantly the capacity of the liver of newborns to oxidize tyrosine as soon as 2 hours after birth (*4*).

In newborns, other means have been utilized to produce alterations in normal developmental patterns. For example, development of hepatic glucokinase in weanling rats can be induced or repressed by the glucose content of the diet (*5*); and simulated altitude conditions affect the activity as well as the isozyme patterns of lactate dehydrogenase in neonatal rats (*6*). Also, exposure to hypoxia for 12 and 24 hours increases the activity of glycolytic enzymes in newborn rabbits (*7*).

In normal rats, appreciable tryptophan oxygenase activity does not appear until day 12 after birth, while animals treated with L-tryptophan demonstrate hepatic tryptophan oxygenase activity as early as day 3 (*8*). In addition, injection of hydrocortisone produces significant tryptophan oxygenase activity in 7-day-old rats (*9*). Because exposure of adult animals to hypobaric hypoxia results in the induction of tryptophan oxygenase (E.C. 1.13.1.12, L-tryptophan : oxygen oxidoreductase) in the liver (*10*), we sought to determine whether this stress would similarly enhance the development of this enzyme in neonatal rats.

Sprague-Dawley rats (Charles River Breeding Laboratories) at the same stage of gestation were placed in separate cages with adequate nesting material and free access to food and water. All animals were housed at 21° ± 2°C in rooms having a 12-hour light (0600 to 1800 hour) period followed by a 12-hour dark (1800 to 0600 hour) period. At least 4 days before scheduled parturition, the experimental animals were brought to a simulated altitude of 5790 m (oxygen pressure, 76.36 mm-Hg) in closed Plexiglas chambers partially evacuated by a vacuum pump. Barometric pressure was controlled by an adjustable air-inlet valve. The animals remained at this simulated altitude through the remainder of pregnancy and birth, and throughout the postpartum period, with return to sea level only to remove neonatal animals. Control animals maintained at sea level were housed in the same room under the same cage conditions. Neonatal rats were weighed and killed; the livers were immediately excised, weighed, and frozen on Dry Ice.

Tryptophan oxygenase activity was assayed with slight modification of the methods of Knox and Auerbach (*11*). Whole homogenates were prepared in 0.14*M* KCl containing 2.5 m*M* NaOH. Methemoglobin (0.5 mg) and L-ascorbate (10 μM) were added to the incubation medium, and the reactions proceeded for 1 hour at 37°C under an atmosphere of O_2 and CO_2 (95 : 5) for maximum, consistent enzymatic ac-