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12. We thank Dr. C. Sherr for many helpful comments and criticisms and B. Comfort, L. Lahey, and R. Nalewaik for technical assistance. Supported in part by the NCI Virus Cancer Program.

15 March 1976

Embryonal Neoplasms in the Opossum: A New Model for Solid Tumors of Infancy and Childhood

Abstract. *Opossums fed the chemical carcinogen ethyl nitrosourea early in postnatal life developed a variety of epithelial and mesenchymal embryonal neoplasms that were closely analogous, in morphology and biological behavior, to tumors of human infancy and childhood for which experimental models in laboratory animals are either imprecise or nonexistent. The embryonal tumors were found in association with, and occasionally at the same sites as, a limited number of malformations.*

Laboratory investigation of embryonal neoplasms (*1*) has been handicapped by the lack of precise experimental models in laboratory animals for the majority of the most important embryonal neoplasms of soft tissue (*2, 3*), bone, and teeth (*1*). Attempts to induce such neoplasms by transplacental exposure of standard laboratory animals to a variety of carcinogens have, with rare exceptions, yielded tumors that do not differ in latency or morphology from nonembryonal tumors induced postnatally with the same agent (*2–4*). The essentially similar response to both transplacental and postnatal carcinogen administration in the traditional laboratory mammals cannot be explained solely on the basis of factors that prevent the carcinogen from reaching the fetus in full concentration or activity—for example, short biological half-life or placental and maternal metabolic alteration or inactivation, or both (*3, 5*). Rice has suggested that another, perhaps more important, reason for the lack of success in induction of embryonal tumors in the common laboratory species, especially for those neoplasms susceptible to endocrine inhibition and immunologic suppression, may be the short interval between the completion of organogenesis and the functional maturation of the reproductive and lymphoid systems (*3*). He also suggests that, in rodents, particularly, the growth of a transplacentally induced tumor would have to be explosive to achieve detectable size before onset of endogenous sex hormone secretion and lymphoid activity.

The postnatal completion of embryonic and fetal development in the opossum (*Didelphis virginiana* Kerr) suggested to us that this species might offer certain advantages over the typical eutherian fetus in utero for experimental oncogenesis and especially for embryonal tumor induction (*6, 7*). At birth, after a gestation of 13 days ± 6 hours (*8*), the opossum represents an amalgam of fetal and embryonic tissue; while determination is largely complete, differentiation has only progressed to the degree necessary for extrauterine survival (*9*). The components of the endocrine system are still in the anlage state (*9*), the gonads are just beginning to differentiate (*10*), the lymphoid system is largely unformed (*11*), and there is no immunologic competence (*12–14*). The first 2½ months of postnatal development in the opossum are roughly equivalent to the last 7 to 8 months of intrauterine development in the human and to the final 8 to 9 days of gestation in the typical rodent (*8–14*). During this period the young opossum is involuntarily attached to the maternal teat within the marsupium, or pouch, where maternal influence is limited to constituents of maternal milk (*15*) and the pouch environment (*16*). Accordingly, when the opossum is used as the experimental animal, it is possible, beginning in some tissues at the anlage state, to expose developing embryonic and fetal tissue to carcinogens in the absence of placental interference, under minimal maternal influence (*6, 7*), and without toxicity to the mother.

Furthermore, the opossum matures slowly in relation to its short life span (about 2½ years) and relatively small body size (about 1.5 to 3 kg). For example, maturation of the lymphoid system requires about 60 days after birth (*11*), and full sexual development is not attained for at least 9 months after birth (*8*). Demonstrated immunologic consequences of the lymphoid maturation rate in the opossum are: (i) the absence of circulating antibody production before 7 days of age (*12*), (ii) an inability to reject skin allografts prior to 12 days of age (*13*), and (iii) an acquired tolerance to soluble and particulate antigens administered before 2 weeks of age (*14*). Thus, there is more time in the opossum than in the typical laboratory mammal for the oncogenic process to proceed prior to the full functional maturation of several factors that may influence or inhibit the oncogenic chain of events.

In two separate experiments performed in successive years, 532 opossums from 72 litters bred in captivity (*17, 18*) and obtained within 17 hours of birth (*18*) were divided by litter into ten groups according to age (< 1 day old and 1, 2, 3, 4, 6, 8, 10, 12, and 16 weeks old). In the first experiment (*7*), animals were given approximately 100 mg of ethyl nitrosourea (ENU) (*19*) per kilogram of body weight as a single dose by mouth. In the second study the same total oral dose of ENU was given every other day in four increments of 25 mg per kilogram of body weight. The ENU was administered within 1 hour of solution in *p*H 4.0 saline phosphate buffer. One or two animals in each litter were given saline phosphate buffer only. Opossums with visible tumors were killed at various ages, depending on the condition of animal and tumor. Animals without grossly manifest tumors were killed either when moribund or 2 years after drug administration.

A variety of mesenchymal and epithelial neoplasms, including embryonal neoplasms of the eye, liver, brain, kidney, muscle, and jaw, developed in the opossums treated with ENU. Three of the embryonal tumors, an intraocular neoplasm (teratoid medulloepithelioma), a tumor of the neuron (ganglioglioma), and an odontogenic tumor of the jaw (myxoma) have not, to our knowledge, been previously induced by systemic administration of a carcinogen. In this report we present preliminary data on the susceptibility of the opossum to induction of embryonal tumors; more detailed statistical and descriptive data on the induced neo-

plasms, both embryonal and nonembryonal, are available (*20*).

Unilateral, malignant, neuroectodermal intraocular tumors (Fig. 1, A to C), analogous in morphology and behavior to the teratoid medulloepithelioma (diktyoma) of the human (*21*), developed in five animals (two animals were littermates) that were exposed to ENU between 7 and 21 days of age. In man these tumors are closely related to the retinoblastoma with origin from embryonic retinal elements in the ciliary body. The intraocular tumors in the opossum, like those in man, became clinically apparent early in postnatal development and did not metastasize but, rather, grew to fill the eye (Fig. 1A), causing eventual rupture of the globe and sloughing of the intraocular content. A neuroectodermal component with neuroepithelial and neuroglial differentiation (Fig. 1B and inset) was predominant. A scanty mesenchymal element, which showed differentiation to four types of heteroplastic tissue (Fig. 1C), accounted for the teratomatous nature of the lesion.

Embryonal tumors of the kidney, morphologically identical to the human nephroblastoma (Wilms' tumor) (*22*, pp. 51–57; *23*), were found in approximately 30 percent of opossums that were exposed to ENU within 6 weeks after birth. Like nephroblastomas in infants and children, but unlike the usual experimental tumors designated as nephroblastomas (*3*), the kidney tumors in the opossum were often massive (up to 325 g, or about 20 percent of the body weight of the host animal) (Fig. 1D), occasionally metastasized, and contained neoplastic counterparts of both the mesenchymal and the epithelial elements (Fig. 1E) of the fetal kidney.

Although there was a high incidence of malignant cholangiocarcinomas and liver cell tumors in the opossums treated with ENU, only one animal treated at birth developed an embryonal neoplasm of the liver (Fig. 1F). This tumor, which was morphologically similar to neoplasms described in mice treated with DDT (*24*), was composed of neoplastic cells with small round hyperchromatic nuclei and scanty, darkly basophilic cytoplasm arranged either around large vascular channels or in configurations resembling rosettes, in sheets, or in ribbons (Fig. 1G).

Several forms of neurogenic neoplasms, including two types of embryonal brain tumors, were identified in opossums exposed to ENU within 8 weeks of birth. A tumor of the cerebral hemisphere, morphologically identical to the ganglioglioma of children and young adults (*25*), was seen in eight animals exposed to ENU on the day of birth and in each of two animals treated at 1 week and 8 weeks, respectively. As in man, this tumor in the opossum grew slowly and showed a spectrum of neuronal maturation (Fig. 1H), ranging from neuroblasts to bizarre multinucleated neurons (Fig. 1H, inset) within a glial matrix of varying density. Experimental induction of the ganglioglioma is of interest because this neoplasm alone among brain tumors has not previously been induced experimentally.

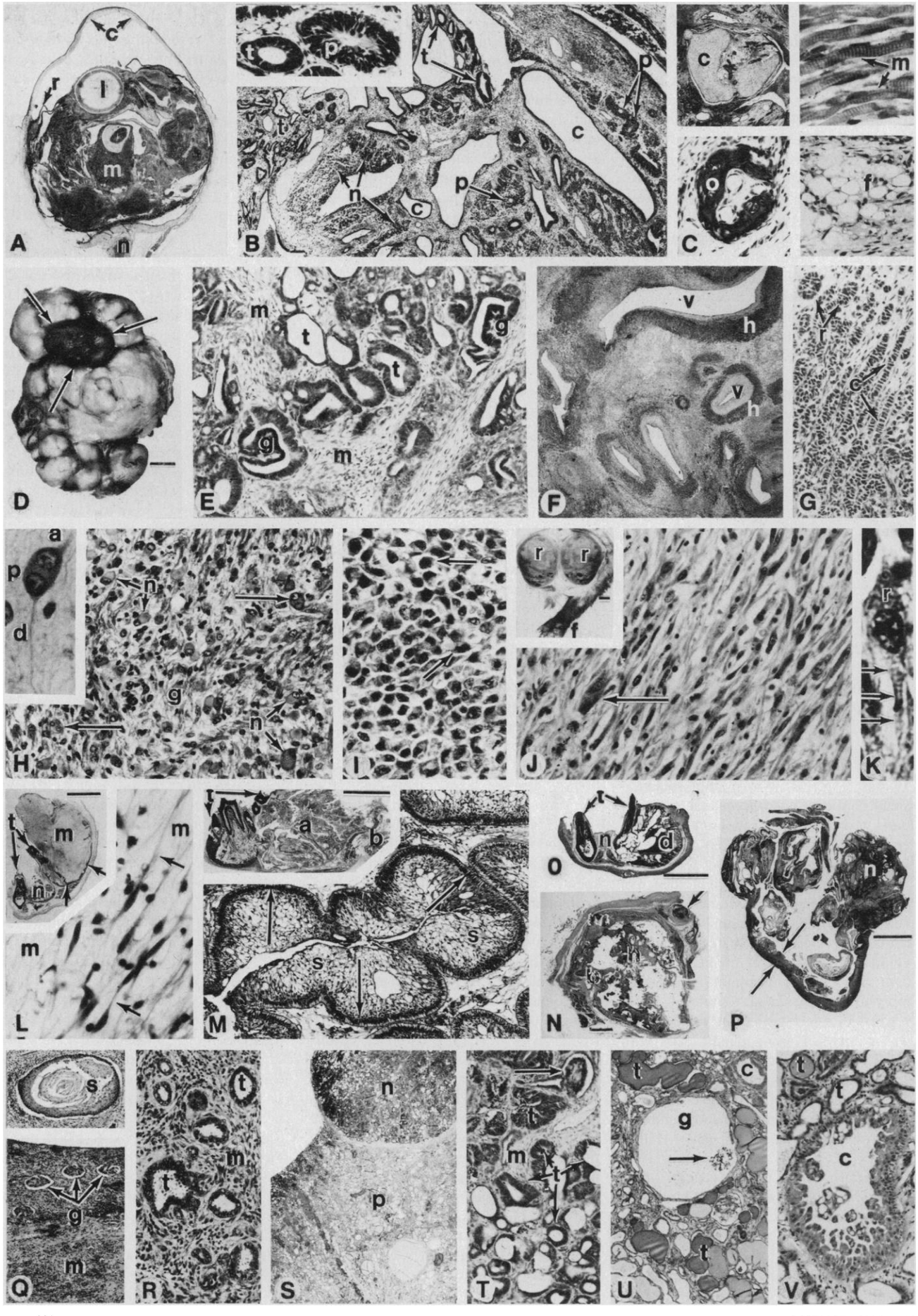
A second type of neurogenic tumor, analogous to the so-called primitive neuroectodermal tumor of children (*26*), was seen in the cerebral hemispheres of two animals. These neoplasms were composed essentially of undifferentiated cells resembling germinal or matrix cells of the embryonic neural tube (Fig. 1I), with focal areas of glial and neuronal differentiation.

Several different types of mesenchymal tumors of soft tissue were found in opossums given ENU at various stages of postnatal development. Three of these tumors, a sarcoma of the maxilla in two animals and a sarcoma of the thigh in one animal, were induced before 1 month of age and showed a resemblance to muscle tumors of embryonal origin. The tumor of the thigh is of particular interest because of its resemblance to an embryonal tumor of striated muscle. This lesion, which attained massive size (Fig. 1J, inset), was composed of pleomorphic and stellate cells associated with occasional bizarre multinucleated giant cells (Fig. 1J). Rare neoplastic cells with cross striations (Fig. 1K) confirmed the diagnosis of rhabdomyosarcoma, a malignant neoplasm believed to arise from either prospective muscle or undifferentiated mesenchymal cells (*1*; *22*, pp. 67–75).

Both epithelial and mesenchymal embryonal tumors were induced in the jaws of opossums given ENU through 12 weeks of age. A mesenchymal tumor (myxoma) of the mandible (Fig. 1L, inset) was present in each of two opossums treated with ENU at birth. This tumor, which was composed of neoplastic stellate cells with fibrillary processes in a mucinous matrix (Fig. 1L), is believed to originate either from the anlage of the teeth or from osteogenic mesenchyme in the jaw (*27*). The most common epithelial tumors of the jaws were aggressive odontogenic neoplasms of the maxilla and mandible with a close morphological and biological resemblance to the human ameloblastoma (Fig. 1M, inset). Such tumors were found in 16 opossums given ENU between birth and 12 weeks of age. The presence of classical epithelial configurations resembling the stellate and border epithelium of the developing enamel organ was diagnostic (Fig. 1M). Odontoid differentiation, as well as malignant transformation, was present in individual tumors. In the opossum, the longer susceptible age range for ameloblastomas in comparison to the other induced embryonal neoplasms is in agreement with the theory of origin for these neoplasms in the human—that is, from embryonic remnants either of Hertwig's sheath or the enamel organ or from the buccal mucosa (*1*, *28*). The ameloblastomas induced in the opossum with ENU are more analogous to those found in man than are odontogenic tumors induced postnatally with methyl nitrosourea in the continuously erupting canines of the hamster (*29*), since tooth formation is a process of finite duration in both the human and the opossum.

Several types of hamartomas and malformations developed in opossums that were given ENU prior to 3 weeks after birth. These lesions were frequently present in animals affected with tumors, occasionally at the same sites as the tumors. The most frequently seen hamartomatous lesions were ossifying fibrous dysplasias and angiomas. Ossifying fibrous dysplasia of bone was present in 16 opossums given the carcinogen prior to 8 weeks of age. This lesion, which results from a disturbance in osteogenesis (*22*, pp. 131–133), occurred either in the opossum skull or mandible (Fig. 1O), which are also frequent sites of the analogous lesions in children and adults (*30*). Angiomas were found in the skin and liver and occasionally in the epididymis, the left atrium, and the jaw (Fig. 1N). The least common malformation was hypoplasia of the kidney (two cases). The most frequently seen malformations were genital hypoplasia and simple and multiloculated cysts (often massive) of the liver and kidneys. Unilateral dysgenesis of the kidney was found in 12 animals exposed to ENU at 1 week of age. Histopathologic changes resembling those seen in nephroblastoma were present in two of the dysgenetic kidneys (Fig. 1, P to R). Renal malformations resembling polycystic kidneys were seen bilaterally in four animals treated with four incremental doses of ENU during the second week after birth. A nephroblastoma as well as multiple cystomas (*31*) were present in one of the cystic kidneys (Fig. 1, S to V).

To our knowledge, experimental induction of tumors at the sites of malformations has not previously been reported in traditional laboratory animals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despite the occasional coincident occurrence of both lesions in the same organ in the human (*32*). Our findings dispute the concept, based on these negative results in standard laboratory species, that "teratogenesis and carcinogenesis within the same organ exclude each other [because] the relevant target molecules, even if nucleic acids, are probably different" (*33*).

Our ability to induce embryonal tumors in the opossum may be explained in part by the unique opportunity in this species for direct exposure of slowly maturing fetal and embryonic tissue to carcinogens in the absence of interference that is attributable, in the eutherian mammal, to the mother, the placenta, and the fetoplacental unit. A more important factor facilitating the induction of such tumors in the opossum may be the slow postnatal maturation characteristic of the marsupial. In particular, the late development of sexual and lymphoid function in the opossum may provide a longer period than is available in the typical eutherian laboratory mammal for the initiation and development of the oncogenic process in the absence of the inhibition that is often associated with full endocrine and immunologic function. That the opossum has an innate susceptibility either to the types of embryonal tumors induced or to the carcinogen, or to both, must also be considered. This possibility is supported by the absence of embryonal tumors, other than in the kidney, in rats transplacentally exposed to comparable doses of ENU (*34*). Species specific responses to carcinogens are well established (*35*), and a hereditary predisposition to certain embryonal neoplasms appears to be present in many species, including man (*36*). A recent taxonomic study which shows that the opossum is karyotypically, and possibly in other ways as well, an aberrent form by comparison to other marsupials (*37*) is pertinent to any genetic considerations. Perhaps also relevant to any explanation for the opossum's susceptibility to embryonal tumors similar to those found in man are experiments which show that the relative effectiveness of certain alkylating agents and mutagens in inducing DNA repair in opossum lymphocytes in vitro is the same as that in human lymphocytes (*38*).

Our results imply that the opossum early in postnatal life is a useful model for the study of certain of the major dysontogenetic neoplasms that have been difficult or impossible to reproduce in the standard laboratory species. A major advantage of the opossum model is that it permits developing tissue to be exposed directly to carcinogens in the absence of indirect effects resulting from reactions that, in utero, may occur between the carcinogen and interposed maternal and placental tissues. Thus the model may be especially useful in exploring the relation between susceptibility to neoplastic transformation and target tissue differentiation (*20*) under conditions where extra-fetal activation or inactivation or a short biological half-life of the carcinogen may obscure or prevent the response of the target tissue. The opossum model may also be of value in clarifying the apparent interrelationship of carcinogenesis, teratogenesis, and mutagenesis in vivo, since it assures that all three processes result either spontaneously or from direct injury to the target tissue.

The susceptibility of the opossum, at stages of development comparable to in utero stages in man and other eutherian species, to a wide variety of organotrophic neoplasms with diverse latencies, provides experimental support for clinical evidence (*39*) of the importance of the perinatal period in cancer induction.

Fig. 1. Neoplastic and teratologic lesions induced in opossums fed ENU early in postnatal life. Stain is hematoxylin and eosin unless otherwise indicated. (A) Calotte of eye containing teratoid medulloepithelioma; *c*, cornea; *r*, iris; *l*, lens; *m*, tumor; *n*, optic nerve (× 4.8) (B) Malignant intraocular teratoid medulloepithelioma; *n*, primitive neuroectodermal component; *c*, cysts; *p*, pseudorosettes; and *t*, tubules representing neuroepithelial differentiation (× 40). Higher magnification of two of the neuroepithelial configurations is shown in the inset; *t*, tubule; *p*, pseudorosette (× 160). (C) Mesenchymal heteroplastic elements in a malignant intraocular teratoid medulloepithelioma. Clockwise beginning at top left: *c*, cartilage (Masson, × 12); *m*, striated muscle (phosphotungstic acid–hematoxylin, × 350); *f*, fat (× 140); *o*, osteoid (× 163). (D) Nephroblastoma, gross appearance. The kidney is indicated by arrows. The scale bar represents 1 cm. (E) Nephroblastoma, microscopic appearance; *t*, neoplastic tubules; *m*, mesenchyme; *g*, pseudoglomeruli (× 85). (F) Hepatoblastoma; *v*, blood vessels; *h*, tumor cells (× 17). (G) Hepatoblastoma; tumor cells are arranged in columns, *c*, and crude configurations resembling rosettes, *r* (× 110). (H) Ganglioglioma of the cerebral hemisphere; *n*, neurons; arrows indicate multinucleated neoplastic cells, probably neurons (× 260). Higher magnification of a binucleated neoplastic neuron is shown in the inset; *a*, axon; *p*, perikaryon; *d*, dendrite (× 520). (I) Primitive neuroectodermal tumor of the cerebrum, the section shows undifferentiated, matrix-like cells. The arrows indicate cells in mitosis (× 282). (J) Mesenchymal tumor of the thigh resembling a rhabdomyosarcoma composed of pleomorphic and spindle-shaped cells and occasional multinucleated giant cells (see arrow) (× 228). The inset shows gross appearance of the tumor, *r*, in hemi section; *f*, leg. The scale bar represents 1 cm. (K) A neoplastic cell, *r*, with apparent cross striations (see arrows) from the tumor shown in (J) (× 1000). (L) Myxoma of the mandible. Neoplastic stellate cells with fibrillary processes (see arrows) are found within a mucinous matrix, *m* (× 400). The inset shows low power appearance of a cross section of the mandible and the relationship of the myxoma, *m*, to the teeth, *t*, and jawbone (arrows); *n*, tongue. The scale bar represents 1 cm. (M) Ameloblastoma of the mandible. Typical configuration of epithelial elements resembling the stellate reticulum, *s*, and border epithelium (arrows) of the developing enamel organ (× 56). The inset shows the low power appearance of a longitudinal section through the mandible containing the ameloblastoma; *t*, teeth; *b*, jawbone; *a*, ameloblastoma. The scale bar represents 1 cm. (N) Massive hemangioma of the jaw. A longitudinal section through the head is shown. The orbit is indicated by an arrow; *m*, muscle compressed by hemangioma; *h*, hemangioma; *b*, jawbone. The scale bar represents 1 cm. (O) Ossifying fibrous dysplasia of the mandible. A cross section through the mandible is shown; *d*, ossifying fibrous dysplasia; *t*, teeth; *n*, tongue. The scale bar represents 1 cm. (P) Dysgenetic kidney with a component resembling nephroblastoma. The functional portion of the kidney is indicated between the two arrows; *n*, nephroblastoma-like component. The scale bar represents 1 cm. (Q) (bottom) A section taken through the functional portion of the dysgenetic kidney shown between the arrows in (P); *g*, glomeruli; *m*, renal medulla (× 27); (top) dysplastic tissue in the form of a squamous cyst, *s*, found in the dysgenetic kidney shown in (P) (× 33). (R) Higher magnification of the nephroblastoma-like component indicated by *n* in (P); *t*, tubules; *m*, mesenchyme (× 100). (S) Polycystic kidney containing a nephroblastoma; *p*, polycystic kidney; *n*, nephroblastoma (×5). (T) Higher magnification of the nephroblastoma shown in (S); *t*, neoplastic tubules; *m*, mesenchyme; arrow, pseudoglomerulus (× 60). (U) Higher magnification of the cystic kidney shown in (S); *t*, cystic tubules; *g*, cystic glomerulus; arrow, glomerular tuft; *c*, cystoma (× 25). (V) Higher magnification of a typical cystoma from the cystic kidney shown in (S); *c*, cystoma; *t*, cystic tubules (× 68).

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40. We thank Dr. J. Alderson, Dr. L. E. Zimmerman, Dr. J. M. Henry, Dr. K. M. Earle, and Dr. C. J. Davis of the Armed Forces Institute of Pathology (AFIP), Dr. H. L. Stewart, Dr. J. M. Rice, Dr. Clyde Dawe, and Dr. W. G. Flamm of NIH, and Dr. B. F. Fetter and Dr. D. Hackel of the Duke University Medical Center for comments and suggestions; L. Miller of AFIP and C. Lewis of Duke University Medical Center for gross photography; R. Nye, W. Sewald, and J. Romine of NIH, J. Edwards and L. Duckett of AFIP, and B. Boyarsky and C. M. Bishop of Duke University Medical Center for photomicroscopy; T. L. Johnson of NIH and the Division of Laboratory Animal Resources at Duke University for animal care; and R. L. Dunn and H. Snells of NIH for technical assistance.

11 December 1975; revised 11 May 1976

Cholinergic Changes During Conditioned Suppression in Rats

Abstract. *Levels of acetylcholine were significantly elevated in the telencephalon and diencephalon + mesencephalon of rats killed by near-freezing during conditioned suppression of food-reinforced lever pressing, whereas levels of serotonin, dopamine, and norepinephrine were not altered. These neurochemical changes were not seen in rats serving as controls for conditioning experience, activity levels, or stimulus presentation.*

In a series of studies on drug-induced atypical behavior in rats, a special type of behavioral excitation was found to be temporally related to decreased total levels of acetylcholine (ACh) in the telencephalon (*1*), whereas depressed responding was accompanied by increased levels of this putative transmitter in the diencephalon + mesencephalon (*1, 2*). In both cases, the behavioral states were attributed, at least in part, to changes in release of ACh at cholinergic synapses (*3*).

One disadvantage in using drugs to induce abnormal behavior is the confounding variable of complex interactions among drug, behavior, and neurochemical changes. In addition, drug administration may result in large changes in neurotransmitter levels when relatively small differences may be responsible for the observed atypical behavior. Therefore, to provide more precise correlations between behavior and neurotransmitter levels, it is also necessary to use nondrug methods for altering behavioral states. The conditioned emotional response (CER) procedure has been used extensively to produce conditioned suppression without the use of drugs (*4, 5*), and provides one method for investigating neurochemical correlates of substantial decreases in rates of responding. We report here that levels of ACh are elevated in certain areas of the brain during conditioned suppression of food-reinforced behavior in the rat.

Four groups of adult, male albino rats (Wistar strain) were trained to press a lever for condensed milk in an operant conditioning chamber that contained a lever, a dipper for presentation of liquid reinforcement, a grid floor, and a speaker for presentation of auditory stimuli. The rats were maintained at 85 percent of their free-feeding weights. Once the lever-pressing response was established, the rats were given daily sessions on a variable interval 1 (VI 1) schedule of reinforcement in which 0.15 ml of milk was presented to the responding animal on the average of once per minute. During each session 30 reinforcements were delivered over a period of about 30 minutes.

After levels of stable responding were reached (mean response rate, 42 per minute), the rats continued to receive daily VI 1 sessions. However, at another time of day, at least 2 hours after a VI session, three groups of rats received CER training, whereas a fourth group received only auditory stimulus (S) training. The CER or S training sessions were given in a grid floor apparatus which contained no lever or dipper. The CER training procedure was a modification of the method of Hunt and Brady (*4*). A CER session consisted of a 15-minute period in which six electric grid shocks (2.0 ma, 0.5-second duration) were presented, with each shock preceded by an average of 2 minutes of white noise. The presentations of white noise and shock were interspersed with periods of silence. Defecations or urinations (or both) occurred during 98 percent of the CER sessions. The S training followed the same procedure as the CER training except that no shocks were ever given. In only 5 percent of these latter sessions were defecations or urinations noted. Both CER and S training sessions were given once per day for 7 days, with the final session in a lever-pressing apparatus (again at least 2 hours after a VI session).

On the day after the final CER or S training session, two groups of CER trained rats were placed in the lever-pressing apparatus as usual and allowed to work on the VI schedule. After 5 minutes of responding, the first group was presented with 15 minutes of continuous white noise (which was never followed by shock). The rats were then quickly removed from the chambers and killed by the near-freezing method (*6*). The brains were removed and dissected (*6*) and the parts assayed (*7*) for ACh, serotonin (5-HT), dopamine (DA), and norepinephrine (NE). The second group of CER rats was killed after 20 minutes of working on the VI schedule, never having received white noise during the VI session. The final CER group did not receive any VI experience on the day they were killed, but were killed at comparable times after removal from their home cages following the ingestion of an amount of condensed milk equivalent to that consumed by the first CER group. The S trained rats were presented with the white noise after 5 minutes of working on the VI schedule