Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6966–6970, August 1991
Neurobiology

Transfer of genetic epilepsy by embryonic brain grafts in the chicken

(avian embryonic chimeras/brain tissue transplantation/photic epilepsy)

MARIE-AIMEE TEILLET*†, ROBERT NAQUET‡, GILDAS LE GAL LA SALLE‡, PHILIPPE MERAT§, BERNADETTE SCHULER*, AND NICOLE M. LE DOUARIN*

*Institut d'Embryologie Cellulaire et Moléculaire du Centre National de la Recherche Scientifique et du Collège de France, 49bis, Avenue de la Belle Gabrielle, 94736 Nogent-sur-Marne Cédex, France; ‡Laboratoire de Physiologie Nerveuse du Centre National de Recherche Scientifique, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France; and §Laboratoire de Génétique Factorielle Centre National de Recherches Zootechniques, Institut National de la Recherche Agronomique, 78350 Jouy-en-Josas, France

Contributed by Nicole M. Le Douarin, May 17, 1991

ABSTRACT **In the Fayoumi chicken, a spontaneous recessive autosomal mutation (F.Epi) is responsible for high susceptibility to seizures that are especially inducible by intermittent light stimulation. Substitution of defined areas of the encephalic neuroepithelium in normal chicken embryos at 2 days of incubation by their counterparts from homozygous F.Epi embryos generates the epileptic phenotype in the chimeras. It was found that grafting primordia of both prosencephalon and mesencephalon of homozygous F.Epi birds is necessary and sufficient for transfer of the full disease. When grafted alone, the homozygous F.Epi prosencephalon, although showing the typical epileptic interictal electroencephalogram, does not allow the complete epileptic seizures to occur in the hosts. Grafts of mesencephalon and/or rhombencephalon modify neither the behavior nor the electroencephalographic pattern of the recipient chickens. Cooperation of forebrain and midbrain activities is therefore required to yield epileptic seizures in this model.**

Epilepsy, a well-characterized disease of the nervous system, is highly heterogeneous in its symptomatic manifestations and its etiology. In humans, certain epilepsies clearly result from localized or diffuse alterations of the brain, while the origin of others is difficult to assess. Some forms of human cryptogenic epilepsies, however, have a genetic origin (1–9). Various mammalian models of genetic forms of epilepsy exist and have led to a more precise knowledge of the brain structures involved in the disease (10–13).

A line of chickens in which typical and reproducible seizures can be easily induced by intermittent light stimulation (ILS) has been established in the Fayoumi strain (14–18). The Fayoumi epileptic (F.Epi) mutation is controlled by a single recessive autosomal gene with complete penetrance. This avian model provides specific interesting features related to the possibility of undertaking embryonic manipulations not feasible in mammals, such as the production of brain chimeras (19, 20).

Microsurgical procedures allowing neural chimeras to be constructed were developed some years ago by one of us (21). So far, this method has been applied to interspecific combinations in which neuroepithelial grafts are performed between quail and chicken embryos at day 2 of incubation (E2), prior to the onset of vascularization of the neural tube. This experimental design relies on the ability to distinguish quail and chicken cells by the structure of their nuclei (22), thus providing a cell marking technique to follow migrations of neural crest cells during ontogeny. Recently, such neural chimeras, in which either pieces of spinal cord (23, 24) or of encephalic vesicles (19, 20) were exchanged between quail and chicken embryos, were examined after hatching. Both types of chimeras turned out to be viable and exhibited a sensorimotor behavior compatible with their survival. Species-typical crowing behavior could be transferred from the quail donor to the chicken recipient by means of brain transplants (19). The quail → chicken transplantation system thus leads to functional, albeit chimeric, brains. The limitation of the quail → chicken combinations is that the grafted quail neural tissue is subjected to acute immunological rejection at a variable time after birth. However, we know from the construction of embryonic chimeras involving non-neural tissues that in chicken → chicken combinations virtually no immune rejection takes place, even if donor and host differ at the major histocompatibility complex (25). We could therefore envisage replacing defined territories of the brain vesicles in normal E2 chicken embryos by their counterparts from the F.Epi strain.

The question raised in the experiments related here was to determine whether transplantation of defined regions of the epileptic brain anlage into normal chicken embryos would result in the transfer of the epileptic phenotype. Previous experiments involving embryonic grafts between quail and chicken showed that neural transplantation *per se* never induces epileptic manifestations (19, 20).

MATERIALS AND METHODS

We used chicken embryos of commercial source (JA 57, Institut de Sélection Animale, Lyon, France), known to be nonepileptic, as recipients and homozygous F.Epi or JA 57 (control) embryos as donors of neural epithelium. F.Epi embryonated eggs were obtained from our own breeding by artificial insemination of homozygous F.Epi chickens.

Surgical Procedure. Microsurgery was performed *in ovo* at the 12- to 15-somite stage, when the encephalic vesicles are well defined by constrictions, which were used as limits for the operations. The graft included the neural crest and the superficial ectoderm, as in the case of the brain transplantations previously reported (19, 20). Seven types of experiment were performed (Fig. 1). Six involved the replacement in normal JA 57 chicken embryos of the following regions of the brain by their counterparts from homozygous F.Epi embryos taken at the same developmental stage: experiment I, virtually the whole brain including prosencephalon (with optic vesicles), mesencephalon, metencephalon, and anterior myelencephalon; experiment II, the same brain territories minus

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EEG, electroencephalogram; ILS, intermittent light stimulation; F.Epi, Fayoumi epileptic; Ch.Epi, chimeras constructed for F.Epi analysis; E2, embryonic day 2.

†To whom reprint requests should be addressed.

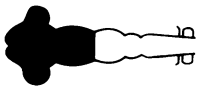
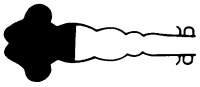
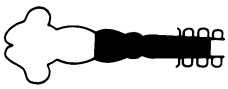
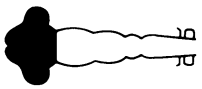
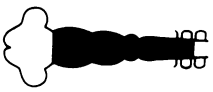
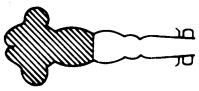
EXPERIMENTS	Ch.Epi	ILS	EEG	Born	Sacrificed
I Pros Mes Met Myel 	1	+	NR	22-5-89	13-9-89
II Pro Mes Met 	2	+	NR	20-6-89	30-10-89
	3	+	Epileptic type	20-6-89	
III Pro Mes 	4	+	Epileptic type	11-1-90	24-7-90
	5	+	NR	22-10-90	
IIIbis Pro 1/2Mes 	6	+	NR	13-11-90	19-11-90
IV Met Myel 	7	−	NR	1-5-90	10-8-90
V Pro 	8	+ / −	Epileptic type	30-5-90	
	9	+ / −	Epileptic type	30-5-90	
	10	+ / −	Epileptic type	5-6-90	15-11-90
	11	+ / −	NR	24-7-90	
	12	+ / −	NR	11-9-90	
VI Mes Met Myel 	13	−	normal	27-6-90	25-8-90
VII Pro Mes 	14	−	NR	28-8-90	5-11-90

FIG. 1. Different vesicles of the brain anlage were replaced microsurgically in E2 normal JA 57 chicken embryos by the equivalent encephalic territories taken either from homozygous F.Epi (experiments I to VI) or from JA 57 (experiment VII) chicken embryos. Shaded areas in diagrams represent regions derived from the donor embryos. Pros, prosencephalon; Mes, mesencephalon; Met, metencephalon; Myel, myelencephalon. We retained for further analysis only those chimeras showing neither morphological nor behavioral defects (Ch.Epi 1 to 14). Video recordings of the chickens' reactions to ILS were made in all cases. Ch.Epi 1 to 6 from experiments I, II, III, and IIIbis responded to ILS by seizure activity (+). Ch.Epi 7, 13, and 14 showed no reaction (−) to ILS, while Ch.Epi 8 to 12 (experiment V) became slightly responsive to ILS (+/−) 2 weeks after hatching but never developed full epileptic seizures. Resting EEGs were recorded in six adult chimeras as well as in homozygous and heterozygous F.Epi chickens and in JA 57 chickens (see Fig. 3). Ch.Epi 3, 4, 8, 9 and 10 (experiments II, III, and V) presented the epileptic-type EEG, like homozygous F.Epi, while Ch.Epi 13 (experiment VI) showed a normal EEG, like that of heterozygous and JA 57 chickens. NR, not recorded. Dates (day-month-year) of hatching (Born) and of sacrifice (Sacrificed) are noted for each chimera.

the myelencephalon; experiment III, the pro- and mesencephalon either complete (experiment III) or deprived of its posterior part (experiment IIIbis); experiment IV, the entire rhombencephalon; experiment V, the prosencephalon alone; experiment VI, the mesencephalon and rhombencephalon. In the last type of experiment (experiment VII), the prosencephalon and mesencephalon of a JA 57 chicken embryo were replaced by the same brain region of an embryo of the same strain.

Video Recordings of Behavioral Responses to ILS. Chimeras and control chickens (homozygous and heterozygous F.Epi and JA 57 animals) were periodically tested under video recordings for seizure susceptibility by using a stroboscopic lamp at 15 flashes per second. ILS was stopped immediately

after initiation of convulsions. When convulsions did not occur, ILS was lengthened up to 3 min in darkness.

Electroencephalogram (EEG) Recordings. For EEG recordings, adult chickens were anesthetized with equithesin (2.5 ml/kg, i.m.) and local analgesia was induced by application of small doses of 1% Xylocaine (lidocaine) at the various pressure points provoked by the stereotaxic frame. Animals were implanted with electrodes for EEG recordings. Five stainless steel jeweler's screws (see Fig. 3) were inserted into the skull on both sides at the anterior and posterior thirds of the cerebrum. Screw 4 served as reference electrode. Screws were then connected to a five-female-pins socket and secured to the skull with dental acrylic cement. EEG recordings were made under resting conditions following at least a 3-day recovery period. Each animal was previously tested for seizure susceptibility with ILS at 15 flashes per second.

RESULTS

Three hundred transplants were performed and 14 viable chimeras (Ch.Epi 1 to 14) were retained for further analysis. We kept only those chimeras presenting neither obvious malformations nor abnormal comportment at hatching (Fig. 1). Their growth rates were similar to those of JA 57 chickens. The chimeras always had feathers with the pigmentation of the donor Fayoumi strain at the level of the graft—i.e., either white or pigmented, but in both cases contrasting with the yellow color of the recipient JA 57 chickens (Fig. 2). Five of these birds (Ch.Epi 1 to 5) presented a typical epileptic phenotype. These belonged to experimental series I, II, and III, involving the graft of at least the forebrain plus the midbrain. As in the case of homozygous F.Epi chickens (14–18), these chimeras underwent seizures from hatching to adulthood, either spontaneously or under ILS. The ILS-provoked seizures were characterized by the stereotyped behavior described by Crichlow and Crawford (15). In phase 1, usually starting within 20 sec after initiation of ILS, the head and neck are first slowly rotated and arched back and upward and the animals make pecking motions with excited vocalizations. Phase 2 is characterized by extension of the wings with some loss of balance. In phase 3, the birds run in all directions, stagger, and then fall on the floor and flap their wings violently with clonic movements of the legs resulting in thrashing and trumbling motions. Phase 3 may continue for several minutes. After the seizure, the chimeras, like the homozygous F.Epi controls, were prostrate and showed transitory wing paresis. Thereafter they progressively recovered a normal behavior. One bird in which the prosencephalon was grafted with only the anterior part of the mesencephalon (Ch.Epi 6, experiment IIIbis) had convulsions under ILS a short time after hatching. Experiment VII, in which both pro- and mesencephalon were exchanged between two embryos of the JA 57 strain, gave rise to a bird (Ch.Epi 14) showing no clinical sign of epilepsy.

The birds in which the rhombencephalon alone (experiment IV) or even both the rhombencephalon and the mesencephalon (experiment VI) of a homozygous F.Epi embryo were implanted (Ch.Epi 7 and 13) never developed epileptic seizures.

Five chickens (Ch.Epi 8 to 12) carried grafts of the homozygous F.Epi prosencephalon alone (experiment V). In contrast to the birds of experiments I, II, and III, they showed no reaction to ILS during the first 2 weeks after hatching. Thereafter, however, they all responded to ILS

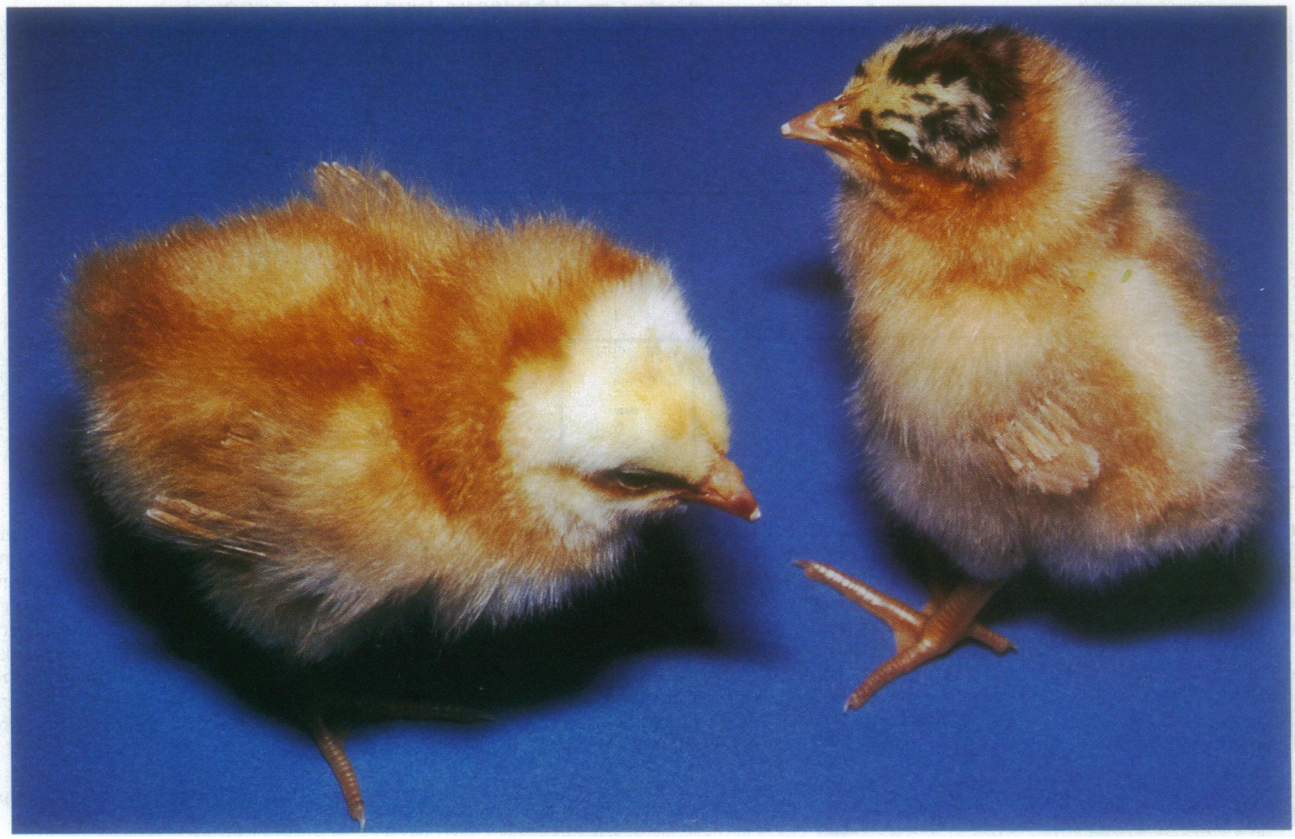


FIG. 2. Ch.Epi 2 (left) and Ch.Epi 3, three days after hatching. These chimeras were constructed by transplanting jointly into E2 normal chicken embryos the pro-, mes-, and metencephalon extirpated from homozygous F.Epi chicken embryos of same stage (experiment II, see Fig. 1). Host chickens (JA 57 strain) are yellow at hatching, whereas chickens of the Fayoumi epileptic (F.Epi) strain are either white or variegated dark and light brown. The chimeras show the F.Epi pigmentation in the graft area because neural crest cells were implanted together with the brain vesicles.

with a pattern of symptoms analogous to the first and second phases of epileptic seizure (neck arched back and upward and loss of balance) but did not progress to phase 3 as in the typical epileptic seizure described above. At the cessation of ILS, the behavior of these birds returned immediately to its previous state.

EEG recordings were taken in freely moving, awake non-epileptic and epileptic adult chickens during resting periods and ILS stimulations but never during the full seizure, to avoid interfering artifacts (Fig. 3). In both JA 57 and Fayoumi heterozygous carrier chickens, the EEG records were normal and made up of low-amplitude rhythms. A similar pattern was found in Ch.Epi 13, which received a graft of mesencephalon plus rhombencephalon (experiment VI). In Ch.Epi 3 and 4 (experiments II and III), which exhibited clinical signs of epilepsy as in homozygous F.Epi chickens, the interictal records were characterized by high-amplitude continuous asynchronous slow waves, slow spikes, or spikes and waves. These abnormalities were prominent when the animals were relaxed. After any stimulus inducing an arousal reaction, the record was transiently constituted by low-amplitude fast rhythms. ILS produced a similar effect during phase 1 of the seizure, but rapidly the symptoms of the seizure itself did not allow EEG activity to be distinguished from muscle spikes and movement artifacts. Immediately after the seizure, the EEG activity was depressed with bursts of slow waves interrupted by silences. Progressively, the amplitude, frequency, and shape of the waves returned as before the seizure.

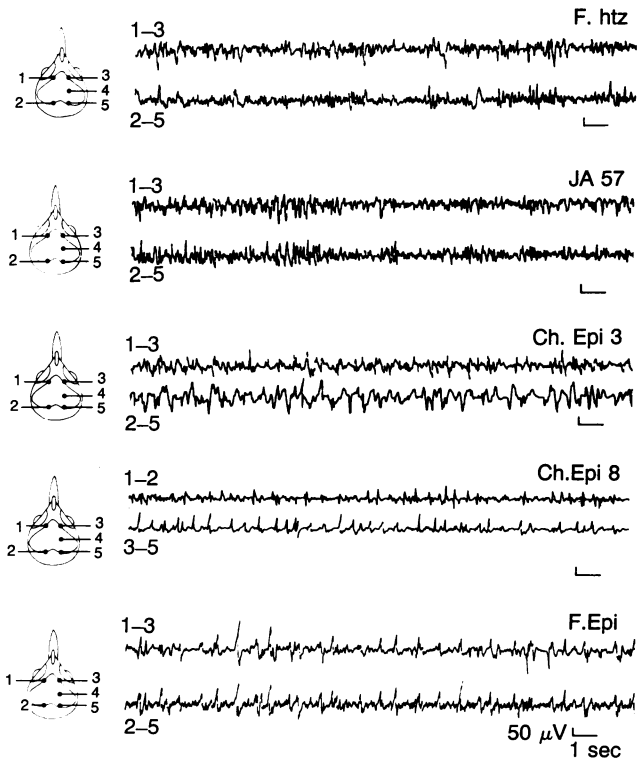


FIG. 3. Resting EEG of a Fayoumi heterozygous chicken (F.htz); a JA 57 chicken, of the recipient strain; two chimeras [Ch.Epi 3 (experiment II) and Ch.Epi 8 (experiment V)]; and a homozygous F.Epi chicken. Note the normal resting EEG in the F.htz and JA 57 birds as compared with the continuous high-amplitude spikes, polyspikes, and slow wave discharges recorded in the homozygous F.Epi bird and the two chimeras. Although the characteristic spikes and spikes and waves have been recorded in Ch.Epi 8 and in other chickens of experiment V (see Fig. 1), chimeras of this series presented symptoms of only phases 1 and 2 of the F.Epi seizure and never the full seizure. Electrode positions are indicated in the diagram shown to the left of each pair of traces.

Interestingly, recorded animals carrying the graft of a prosencephalon alone (Ch.Epi 8, 9, and 10, experiment V) had a resting EEG similar to that of homozygous F.Epi chickens and of Ch.Epi 3 and 4. Moreover, during the symptoms analogous to phases 1 and 2 of epileptic seizure induced by ILS, the EEG showed only a blockade of the paroxysmal abnormalities constituting the EEG background of the homozygous F.Epi chickens. The EEG returned to the previous state immediately after cessation of ILS.

Eight chimeras were sacrificed (Fig. 1). Their brains were dissected and found similar to those of controls, without any detectable malformation, thus showing the perfect integration of the graft into the host's nervous system. The others (Ch.Epi 3, 5, 8, 9, 11, and 12) were still alive (see Fig. 1). Ch.Epi 3 (1½ years old) had seizures well controlled by an adapted treatment with phenobarbital (16).

DISCUSSION AND CONCLUSIONS

These experiments demonstrate that the transfer of a pathological genetic trait affecting the nervous system is possible through *in situ* transplantation of neuroepithelium during embryonic life.

After showing that transplantation of the whole brain [i.e., the four primitive encephalic vesicles (experiment I)] from an epileptic embryo to a normal embryo leads to the transfer of the full disease, implantation of selected regions of the encephalic vesicles provided the opportunity of investigating the role of various neuroepithelial territories in the establishment of the seizure phenotype. We have found so far that only Ch.Epi birds into which at least both pro- and mesencephalon have been implanted presented the full spectrum of the epileptic manifestations. In contrast, the bird implanted at the level of the rhombencephalon (i.e., metencephalic and myelencephalic vesicles, Ch.Epi 7) did not show the epileptic phenotype, nor did that in which rhombencephalon together with mesencephalon were grafted (Ch.Epi 13). Up to their sacrifice (at ages of about 2½ and 2 months, respectively), they showed no sign of seizure activity, neither spontaneously nor under repeated ILS. In contrast, they behaved in these circumstances like normal JA 57 chickens. These results, together with others previously reported (19, 20) and the control experiment [grafting of a JA 57 pro- plus mesencephalon (Ch.Epi 14)], confirm that grafting by itself does not generate epileptic seizures, at least as long as it results in normal brain development.

The birds with prosencephalic grafts, although exhibiting typical interictal paroxysmal EEG, showed only very mild manifestations under ILS; if they presented epileptic fits, they exhibited only the first and second phases of the seizure, which ceased with the withdrawal of the stimulation. On the other hand, the presence of the mutant mesencephalon (transplanted together with the rhombencephalon in Ch.Epi 13) was not sufficient to induce epileptic manifestations. It was only when at least prosencephalon and anterior mesencephalon were grafted together that the complete neural disease was transmitted from a mutant to a normal chicken.

The chimera in which both prosencephalon and anterior mesencephalon were grafted (Ch.Epi 6) is particularly interesting. We know from the analysis of quail $\rightleftharpoons$ chicken brain chimeras that the caudal half of mesencephalon participates in the formation of cerebellum (20). It is striking to see here that transplantation of the rostral half of the mesencephalon together with the prosencephalon appears to be necessary and sufficient to induce the full epileptic phenotype. Therefore, the neuroepithelial territory yielding the cerebellum and characterized by expression of the Engrailed gene (26, 27) seems to be excluded from the epileptogenic area of the brain in this system.

In conclusion, besides the fact that the F.Epi strain constitutes an interesting model to study genetic and pharmacological aspects of "generalized epilepsy" (see ref. 28 for a review), we think that the experimental model developed here may be a valuable means for investigating the importance of certain discrete zones of the brain in eliciting the disease in the genetically normal nervous system and body of the recipient. It is demonstrated here that neither the presence of the epileptic forebrain characterized by typical interictal epileptic EEG nor the presence of a genetically epileptic midbrain is a sufficient condition for a complete epilepsy pattern to occur in this model. A cooperation of these two brain areas is necessary to generate the full epileptic phenotype of the homozygous F.Epi chickens.

We are grateful to Dr. R. Crawford for sending us eggs of the mutant epileptic strain that he discovered and to G. Coquerelle for establishing in the Institut National de la Recherche Agronomique (Jouy-en-Josas, France), a colony of the Fayoumi epileptic chickens and providing us with homozygous breeders. We thank E. Bourson, S. Gournet, B. Henri, Y. Rantier, and S. Roy for their help in the preparation of the manuscript and Profs. Jerome Engel, Jr. (University of California, Los Angeles) and Charles Ordahl (University of California, San Francisco) for critical reading of the article. This work was supported by Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Association pour la Recherche coutre le Cancer, and Fondation pour la Recherche Médicale.

1. Andermann, E. (1982) in *Genetic Basis of the Epilepsies*, eds. Anderson, V. E., Hauser, W. A., Penry, J. K. & Sing, C. F. (Raven, New York), pp. 355–374.
2. Andermann, E. (1985) in *Genetic Aspects of Human Behavior*, eds. Sakai, T. & Tsuboi, T. (Igakuhoin, Tokyo), pp. 129–145.
3. Nekhorocheff, M. I. (1950) *Rev. Neurol. (Paris)* **83,** 601–602.
4. Metrakos, J. D. & Metrakos, K. (1969) in *Basic Mechanisms of the Epilepsies*, eds. Jasper, H. H., Ward, A. A. & Pope, A. (Little, Brown, Boston), pp. 700–708.
5. Doose, H., Gerken, H., Hien-Volpet, K. & Voelzke, E. (1969) *Neuropädiatrie* **1,** 56–73.
6. Doose, H. & Gerken, H. (1973) *Neuropädiatrie* **4,** 162–171.
7. Naquet, R. (1975) in *Growth and Development of the Brain*, ed. Brazier, M. A. B. (Raven, New York), pp. 219–230.
8. Delgado-Escueta, A. V., Greenberg, D. A., Treiman, L., Liu, A., Sparkes, R. S., Barbetti, A., Park, M. S. & Terasaki, P. I. (1989) *Epilepsia* **30,** Suppl. 4, S8–S18.
9. Leppert, M., Anderson, V. E., Quattlebaum, T., Stauffer, D., O'Connell, P., Nakamura, Y., Lalouel, J.-M. & White, R. (1989) *Nature (London)* **337,** 647–648.
10. Killam, K. F., Killam, E. K. & Naquet, R. (1967) *Electroenceph. Clin. Neurophysiol.* **22,** 497–513.
11. Seyfried, T. N. & Glaser, G. H. (1985) *Epilepsia* **26,** 143–150.
12. Le Gal La Salle, G. & Naquet, R. (1990) *Brain Res.* **518,** 308–312.
13. Silva-Barrat, C. & Menini, C. (1990) in *Generalized Epilepsy: Neurobiological Approaches*, eds. Avoli, M., Gloor, P., Kostopoulos, G. & Naquet, R. (Birkhäser, Boston), pp. 286–297.
14. Crawford, R. D. (1970) *J. Hered.* **61,** 185–188.
15. Crichlow, E. & Crawford, R. D. (1974) *Can. J. Physiol. Pharmacol.* **52,** 424–429.
16. Johnson, D. D., Davis, H. L., Bailey, D. G. & Crawford, R. D. (1977) *Can. J. Physiol. Pharmacol.* **55,** 848–854.
17. Johnson, D. D., Davis, H. L. & Crawford, R. D. (1979) *Fed. Proc. Am. Soc. Exp. Biol.* **38,** 2417–2423.
18. Johnson, D. D., Jaju, A. T., Ness, L., Richardson, J. S. & Crawford, R. D. (1981) *Can. J. Physiol. Pharmacol.* **59,** 144–149.
19. Balaban, E., Teillet, M.-A. & Le Douarin, N. M. (1988) *Science* **241,** 1339–1342.
20. Hallonet, M., Teillet, M.-A. & Le Douarin, N. M. (1990) *Development* **108,** 19–31.
21. Le Douarin, N. (1982) *The Neural Crest* (Cambridge Univ. Press, Cambridge, U.K.).
22. Le Douarin, N. (1969) *Bull. Biol. Fr. Belg.* **103,** 435–452.
23. Kinutani, M. & Le Douarin, N. (1985) *Dev. Biol.* **111,** 243–255.
24. Kinutani, M., Coltey, M. & Le Douarin, N. M. (1986) *Cell* **45,** 307–314.
25. Ohki, H., Martin, C., Corbel, C., Coltey, M. & Le Douarin, N. M. (1987) *Science* **238,** 1032–1035.
26. Gardner, C. A., Darnell, D. K., Poole, S. J., Ordahl, C. P. & Barald, K. F. (1988) *J. Neurosci. Res.* **21,** 426–437.
27. Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. & Goodman, C. S. (1989) *Cell* **58,** 955–968.
28. Johnson, D. D. & Tuchek, J. M. (1987) in *Neurotransmitters and Epilepsy*, eds. Jobe, P. C. & Laird, H. E., II (Humana, Clifton, NJ), pp. 95–114.