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AN AVIAN MODEL OF GENETIC REFLEX EPILEPSY

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INTRODUCTION

Reflex epilepsy was first discovered experimentally. Increasing the excitability of a particular sensory cortex while stimulating the corresponding peripheral sensory receptors results in partial myoclonus, which may or may not be followed by self sustained generalized convulsions (1, 6, 32) synchronous with an electroencephalographic (EEG) afterdischarge (42). Genetic reflex epilepsy (GRE) was later discovered in rats by Morgan and Morgan (31). They found a particular strain of rats, in which complex, high frequency auditory stimulation precipitates convulsions. Successively, it was observed that visual stimulation with repetitive flashes of light produced myoclonus and convulsions in familiarly-predisposed humans (44). Visually induced epileptic manifestations have also been found in Baboons *Papio papio* (20, 21, 22) living in a well-defined habitat; genetic transmission has also been suggested in this case (3). Some of the *Papio papio* also show paroxysmal myoclonus induced by surprise or movement (28). Finally, in 1970 Crawford (8) described an individual chicken of the Fayoumi strain affected by GRE, seizures being induced by complex sensory stimulation and particularly by photogenic stimulation. Such epileptic chickens carry an autosomal recessive mutation; the homozygotes exhibit epileptic fits (Fepi for Fayoumi epileptic) while the heterozygotes (Fhtz) do not.

GRE is a type of epilepsy in which a particular sensory stimulus (the "epileptogenic stimulus") evokes epileptic manifestations only in genetically predisposed subjects. Theoretically, the epileptogenic stimulus can be of any sensory modality, each strain or species being more or less sensitive to a particular one. The epileptic manifestations are variable both within and among individuals, going from a simple paroxysmal electrical discharge, with or without motor disorders, to generalized convulsions with or without electrical discharges (34).

With regard to the genetic transmission of the syndrome, no specific genes have been identified so far. The term "predisposition" underlines our ignorance about spe-

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Dedication: The Authors of this article dedicate their recent work in honor of Professor Ottavio Pompeiano (particularly the first author who has been his pupil and collaborator, and remains his friend).

cific neural mechanisms. The Fepi model of GRE makes a particularly suitable preparation for mechanistic studies, because: i) ILS (intermittent light stimulation) – induced epileptic seizures are consistently obtained in the same animal, and are similar in all the affected animals (the length of the interstimulus interval between light flashes is critical in provoking seizures, and the most effective value can vary among subjects); ii) birds allow *in ovo* construction of brain chimeras and iii) chickens are well suited for genetic disease studies due to their short development time (21 days) and the relative ease of obtaining fertilized eggs from controlled matings.

FEPI EPILEPTIC SYMPTOMS

Recent studies (41, 11, 16, 4) have shown that Fepi epileptic symptoms are evoked with complete penetrance in all homozygotes using either visual or auditory stimulation. The coexistence of complete penetrance for the photogenic as well as audiogenic epilepsy provides a unique model of GRE. This allows a comparison of two types of seizures in the same animal.

During rest (interictal period), the electroencephalogram (EEG) of Fepi and Fhtz individuals are very different (Figs. 1, A1 and B1). Fepi EEGs show continuous spikes, polyspikes and waves (8, 41, 17) not seen in Fhtz EEGs.

In spite of this paroxysmal electrical activity, the motor behavior of the animal is normal.

The most effective epileptogenic stimuli (35) are the ILS at the "magic frequency" of 14/sec [15/sec is the most effective frequency for predisposed humans, while a higher frequency of 25/sec is required in Baboons (20)] and intense sound stimulation (ISS) analogous to the intense and complexe sound effective for rodents (18). Epileptic symptoms are divided into preconvulsive and convulsive stages (11): the former are stimulus locked, meaning that they cease if the stimulus is interrupted, the latter are self sustained, meaning that, once started, they do not cease if the stimulus is interrupted.

Soon after the epileptogenic stimulus is applied, the first motor symptoms are evoked. These are generally associated with behavioral alertness. During ILS, there is opistotonus due to a fine neck myoclonus. When recorded with electromyography (EMG), the myoclonus appears to be synchronous to the frequency of the flickering light. During ISS, there is a paroxysmal running designated as running fit (RF), for the homology with the RF described in rodent audiogenic GRE (18). The animal then displays either generalization of the myoclonus or intensification of RF before the precipitation of violent generalized convulsions. Seizures are followed by a short phase of stupor concurrent with EEG flattening, after which all the signs of the interictal paroxysmal activities are progressively restored. However, the next reflex seizure cannot be induced before a variable lapse of time going from thirty minutes to a few hours.

Of particular interest are the EEG changes: during seizures the interictal paroxysmal discharges are suppressed and replaced by a desynchronized low voltage fast

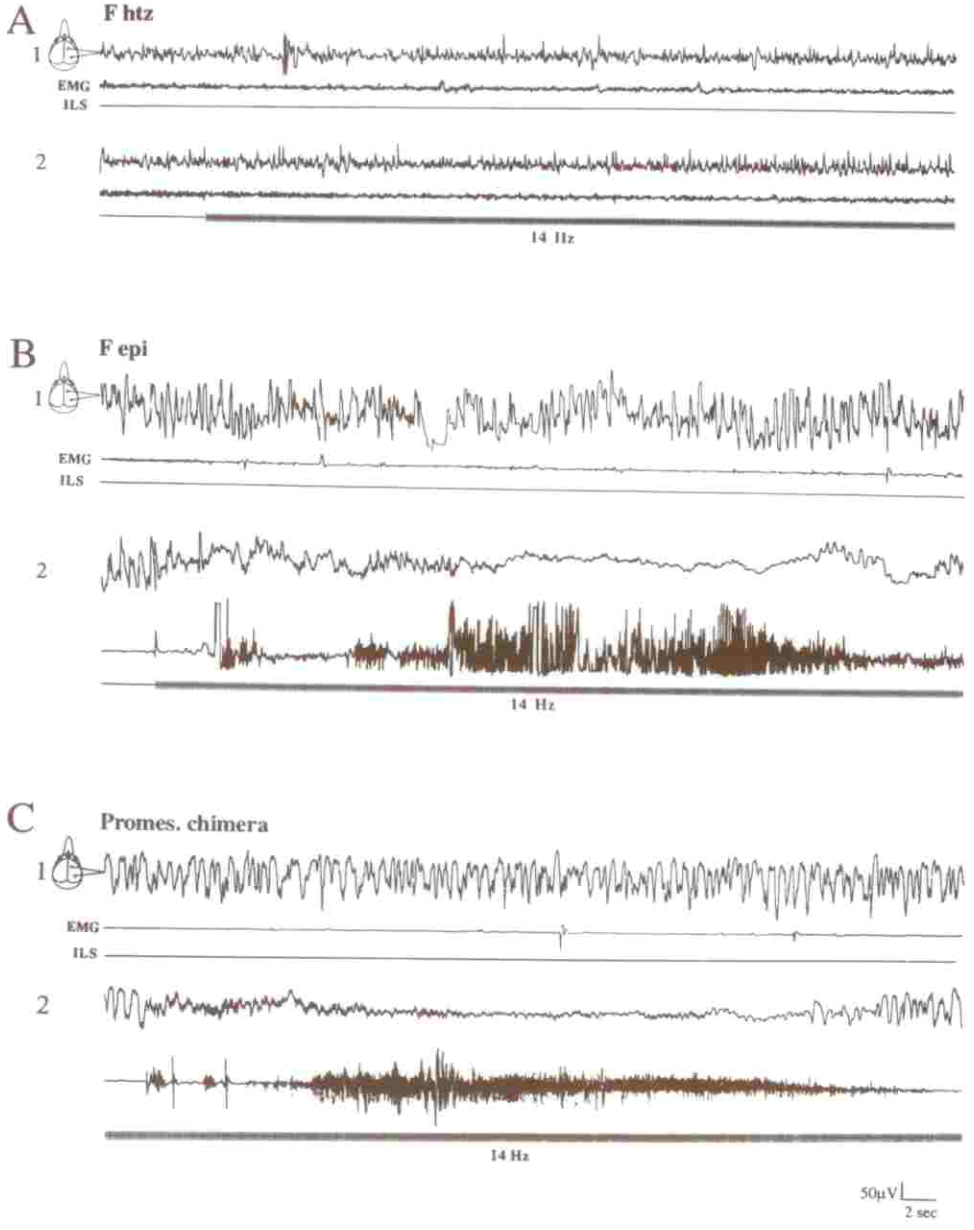


Fig. 1. - *Effects of intermittent light stimulation on Fayoumi chickens and chimeras.*

Recordings of the EEG and the neck muscle EMG in a Fayoumi heterozygous (Fhtz) (A), a Fayoumi homozygous (B) and a group III chimera (C) at rest (1) and during intermittent light stimulation (ILS) (2). Note the ILS-induced seizures in B and C but not in A. Modified from Ref. 17.

activity analogous to the arousal reaction (33), and called "ictal arousal" (17, 11, 34). Ictal arousal starts just before the first paroxysmal behavioral signs, and persists until the motor seizures are completed (Fig. 1, B2).

To understand the behavioral and electrographic paroxysmal symptoms observed in Fepi chickens, extracellular unit activity of the brain structures was recorded at rest and during ILS-induced "EEG ictal arousal" [1]. The activity of two regions was retained (14). *a)* In the prosencephalon, the neurones of the wulst, a visual structure considered homologous to the mammalian visual cortex (19), show bursting activity synchronous with EEG spikes during the interictal periods. This bursting activity, which has never been observed in Fhtz individuals, was suppressed during "ictal arousal" 1 (Fig. 2, A). *b)* In the mesencephalon, on the contrary, unit activity is normal at rest. During the early period of the "ictal arousal" however, the neurones of the Fepi but not those of the Fhtz, show paroxysmal bursting synchronous with the flashing light. This includes visual neurones of the superficial layer of the tectum and tecto-spinal neurones of the deep layer of the tectum. During self-sustained seizures, the ILS-induced tectal bursting disappears, and a long-lasting high frequency activity takes place in a subpopulation of neurons, while others become silent as the EEG is flattening (14). At the late period of the "ictal arousal", all the tectal neurones become silent. Soon after the end of the seizures, unit activities within the prosencephalon and the mesencephalon recover progressively, with a return to the activity patterns typical of the interictal period.

Recordings from Fepi individuals in *ovo* (15) revealed that different symptoms appear at different stages of embryonic development. The resting EEG paroxysmal discharges start on embryonic day 17 (E17), a stage during which functional synapses start to develop in normal chickens (9). Metrazole induced EEG paroxysmal discharges and seizures also start at E17. In contrast, the typical EEG ictal arousal induced by an epileptogenic stimuli is observed on E20 (one day before hatching), a period during which an explosive burst of synaptogenesis takes place (7). No epileptic motor symptoms have ever been observed *in ovo*. Soon after hatching, typical motor seizures, including stimulus-locked symptoms and self-sustained convulsions, are easily evoked by both photic and auditory epileptogenic stimuli.

EMBRYONIC BRAIN CHIMERAS

Embryonic manipulations used to construct chimeras were initiated by Le Douarin who discovered morphologic differences between quail and chick cell nuclei that could be used as cell markers (23). Brain chimeras were obtained by *in ovo* exchanges of a chosen region of the neuroepithelium between quail and chick embryos at E2, before the vascularization of the brain take place (24, 25). Such

[1] For obvious reasons, in this experiment the animals were immobilized with flaxodil. It was previously demonstrated (17) that under flaxedil immobilization, Fepi motor seizures are suppressed but interictal EEG paroxysmal discharges and EEG ictal arousal are maintained.

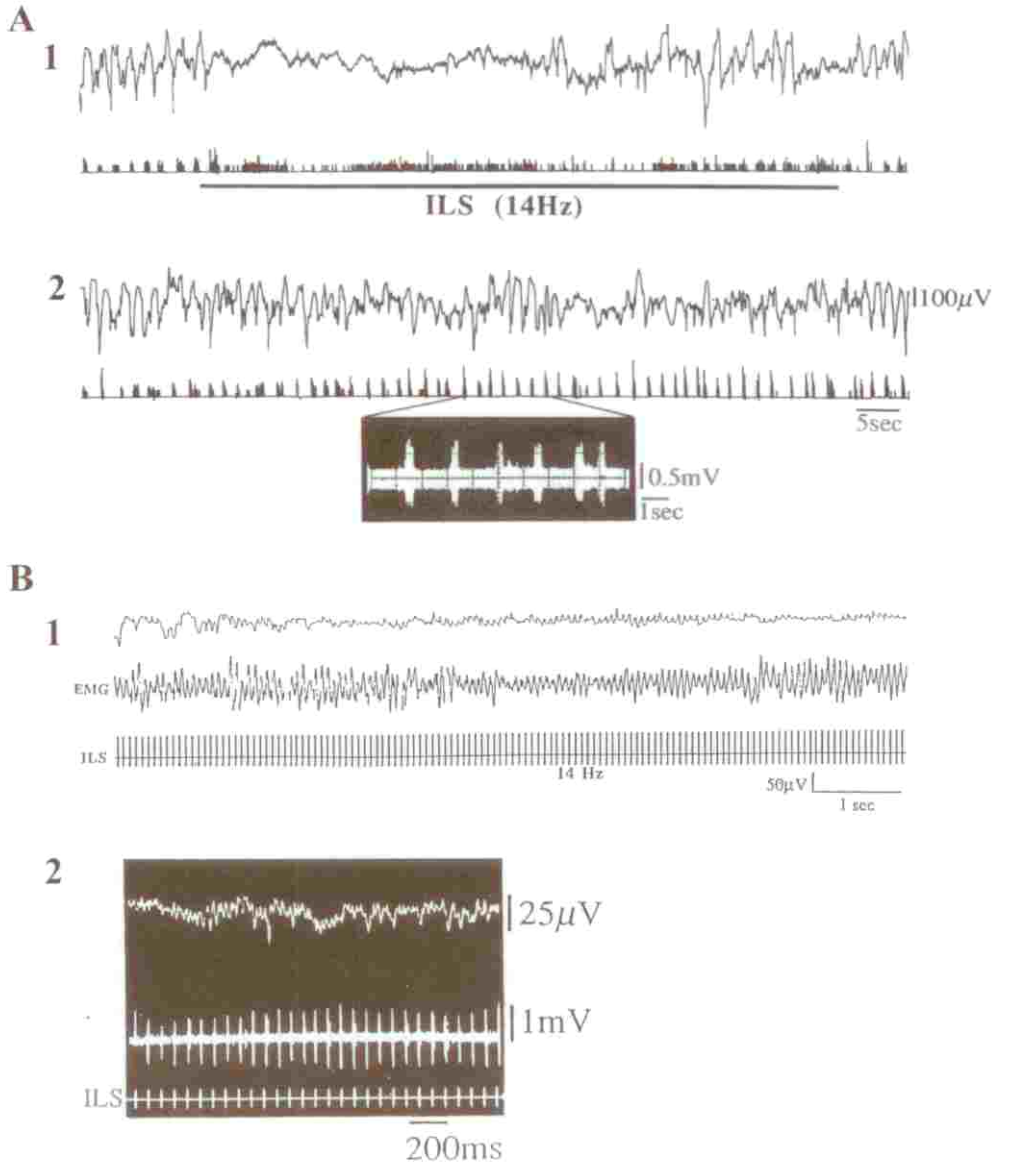


Fig. 2. - *Extracellular unit activities of prosencephalic and mesencephalic neurones in Fepi and chimeras.*

A: Simultaneous recording of EEG (upper trace) and integrated unit activities (lower trace) in the prosencephalon of a flaxedil-paralyzed Fepi during (1) and after (2) ILS-induced ictal EEG arousal. Note the bursting activity of the prosencephalic neurones at rest. B1: EEG and neck muscle EMG recorded in a group III chimera showing persistent neck myoclonus during ILS. B2: Simultaneous recording of the EEG (upper trace) and of a unit in the mesencephalon (middle trace) of a paralyzed group III chimera showing ILS-induced paroxysmal bursting activity of the neurone. Note the normal EEG activity in 1 and 2. Modified from Ref. 14.

chimeras were first used to study the ontogenesis of the nervous system, and subsequently to transfer genetic behavioral traits (24). The idea then came to us to use embryonic brain chimeras to study the Fepi epileptic syndrome (41). The aim of the experiment was to determine whether the epileptic phenotype could be transferred from a homozygous recessive to a wild type embryo by exchanging all or part of the encephalic neural epithelium. Chimeras between Fepi and wild type (commercially-obtained eggs of the JA strain) embryos (Fig. 3) were specifically made to study Fepi GRE. Such chimeras do not exhibit significant immunorejection, and are able to hatch and live to adulthood. The brains of the chimeras were histologically examined *post mortem*, and only results obtained in animals having perfectly normal brain morphology are reported. Several type of chimeras, illustrated in Figure 4, were constructed.

In the first experiment, it was demonstrated that the complete GRE phenotype (including photogenic and audiogenic GRE) could be transferred from a Fepi donor to a normal recipient by substitution of nearly all of the brain vesicles (41). In other words, the epileptic dysfunction is indeed carried by the grafted Fepi neuroepithelium, and is not modified during embryonic and postnatal development in spite of developing in the neural environment of a normal subject. In addition, total transfer of GRE (group II chimeras in Figure 4) was al so obtained with a substitution of the prosencephalic and the mesencephalic vesicles together (Fig. 1, C) (17, 11, 4), implying that they are the minimum amount of neuroepithelium sufficient to reproduce the Fepi phenotype.

In a further step, chimeras containing exchanges of more specific territories of Fepi neuroepithelium were constructed. The idea was to see if it was possible to dissociate different epileptic symptoms in order to know whether they originate in separate brain areas.

It was found that when the prosencephalic vesicle of the Fepi donor was grafted (group I in Figure 4), none of the motor symptoms of the GRE were transferred. Only the electrographic paroxysmal dysfunctions of the prosencephalon but not of the mesencephalon were transferred in the normal chicken. These include: the interictal EEG spikes and waves; the synchronously associated bursting activity of the prosencephalic neurones; the suppression of EEG paroxysms during ILS- and ISS-induced "ictal arousal"; and the concomitant suppression of the bursting pattern of activity of the neurones recorded in the wulst.

Very different results were obtained when the mesencephalic vesicle of the Fepi donor was grafted by itself or together with the neighboring neuroepithelium, in the absence of prosencephalic material (Figure 3 and group III in Figure 4). None of the prosencephalic electrographic paroxysmal activities were transferred whereas, depending on the stimulus, only selected paroxysmal motor symptoms were transferred. Thus, with ILS, long lasting neck myoclonus (Fig. 2, B1) but not convulsions were evoked. Stimulus locked paroxysmal bursting of the tectal neurones was also observed in the superficial as well as in the deep layers, which were synchronous with the ILS (Fig. 2, B2) and therefore also synchronous with the myoclonus. With auditory epileptogenic stimulation, long lasting RF were observed (unit activity was not recorded with this stimulation), which could be followed by generalized con-

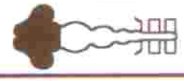
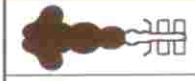
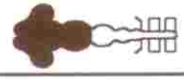
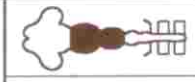
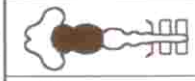
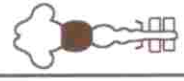
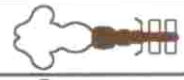
Group	Type of chimera	Number of cases	EEG Paroxysms	ILS				ISS				Type of transfer
				Phase A		Phase B	DF	Phase A		Phase B	D	
				IA	My	Co		IA	RF	Co		
I		3	+	+	—	—	+	+	—	—	+	Transfer of EEG activity
II		1	+	+	+	+	+	+	+	+	+	Total transfer of epileptic phenotype
		3	+	+	+	+	+	+	+	+	+	
III		6	—	—	+	—	—	—	+	+	—	Transfer of ILS-induced neck myoclonus and ISS-induced running fits and generalized convulsions
		2	—	—	+	—	—	—	+	+	—	
		1	—	—	+	—	—	—	+	+	—	
IV		2	—	—	—	—	—	—	—	—	—	No epileptic manifestations
C		1	—	—	—	—	—	—	—	—	—	
		3	—	—	—	—	—	—	—	—	—	

Fig. 3. - *Building a brain chimera.*

A: Schematic representation of the exchanged parts of the neuroepithelium between two chick embryos at the 12-somites stage. The constrictions separating the encephalic vesicles are used as landmarks to limit the graft. In the experiment presented here, the mesencephalic and metencephalic vesicles were excised from a Fepi embryo and grafted in the place of the same structures in a normal chick embryo. The resulting chimera shows the feather pigmentation of the Fepi donor (white) in the graft area, since the neural crest from which pigment cells arise was grafted with the Fepi brain vesicles. Abbreviations for this and the following figure: Co: convulsions, D: EEG desynchronization, DF: EEG desynchronization and flattening. Di: diencephalon, IA: ictal arousal, Mes: mesencephalon, Met: metencephalon, My: myoclonus, Mye: myelencephalon, Pro: prosencephalon, RF: running fit, Rho: rhombencephalon, Tel: telencephalon.

vulsions, although these occurred less frequently then in group II chimeras. In fact, 80% of all sound presentations precipitate convulsions in group II chimeras, versus 30% in group III chimeras. It should be pointed out that the mesencephalon was the minimum amount of Fepi grafted neuroepithelium capable of transferring epileptic symptoms in this group of chimeras.

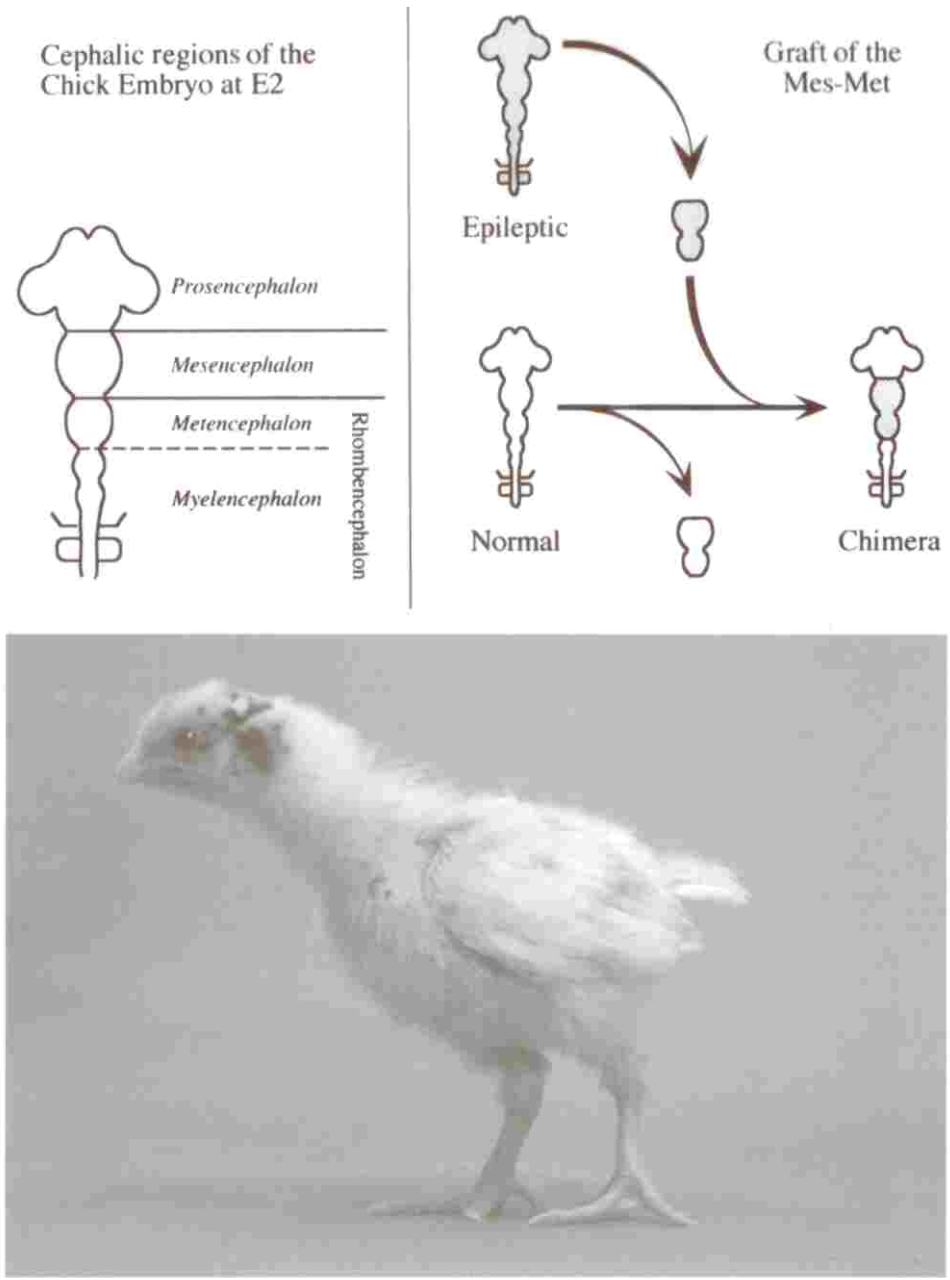


Fig. 4. - *Brain chimeras and transfer of the GRE symptoms of the Fepi.*

Table of ILS- and ISS-induced individual symptoms. The different types of Fepi/JA chimeras were divided into four groups (I to IV) according to the resulting transfer of epileptic manifestations (same group same symptoms). JA/JA chimeras were used as controls (C). The presence (+) or the absence (-) of the transfer of individual symptoms is indicated. See text for explanations. Modified from Ref. 4.

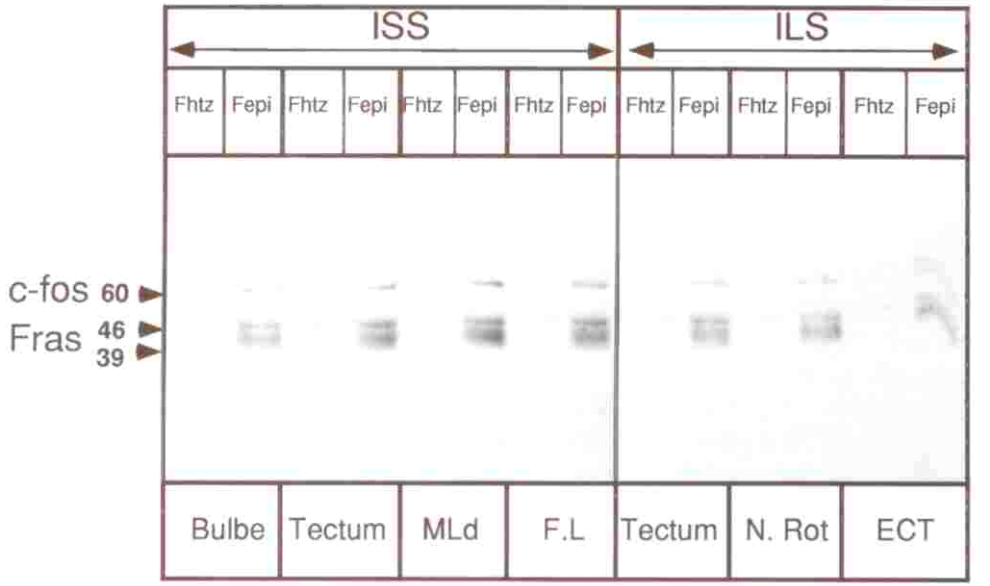


Fig. 5. - *Expression of c-fos in the sensory pathways of the Fhtz and Fepi chickens after epileptogenic stimulation.*

Western blot obtained from two Fhtz and two Fepi sacrificed 3 hours after audiogenic (ISS) and photogenic (ILS) stimulation (seizures were induced in the Fepi and not in the Fhtz). Samples were selected from the same structures in Fhtz (1 to 7) and Fepi (1' to 7'). In the animals submitted to ISS, the samples included primary acoustic relay nuclei of the brainstem *(nucleus coclearis)*, the tectum (deep layers), the *MLd. (nucleus mesencephalicus lateralis, pars dorsalis)* and the FL (field L in the telencephalon). In the animals submitted to ILS, the samples included the visual relays of the tectum (superficial layers), the N. Rot *(nucleus rotundus* in the diencephalon) and the Ectostria (ectostriatum in the telencephalon). Note the increased expression of Fos (60 KD) and FRAs (46 and 39 KD) in the two Fepi chickens.

The last relevant result (41, 17, 4) is that the chimeras having the neuroepithelium caudal to the mesencephalon exchanged (group IV in Figure 4), never produced seizures. This result is consistent with several alternative interpretations. The possibility exists that these parts of the brain do not carry the genetic defect. Alternatively, the genetic defect might be present, but the functions of those brain structures would not be adequate for induction of the epileptic symptoms. As a corollary this would mean that the genetically determined potential to become a Fepi prevails in the anterior part of the brain, not in the posterior part. Finally, these results also show that the motorneurones do not need to have an epileptic genotype to transmit motor seizures.

What we learned from the brain chimeras is that the epileptic phenotype can be transferred in normal chickens totally or partially, depending on the territory of the Fepi neuroepithelium that was grafted. The Fepi prosencephalon by itself only transfers the interictal and ictal electrographic signs of Fepi GRE and therefore only carries a general seizure susceptibility. The mesencephalon transfers neck myoclonus or RF, which are stimulus-locked, and therefore carries only a specific sensory-motor seizure susceptibility. It is through the conjunction of both the prosencepalic

and mesencephalic Fepi neuroepithelium, that ILS or ISS can precipitate or potentiate the appearance of self sustained generalized convulsions. Since neck myoclonus or RF necessarily precedes generalised convulsions, the Fepi mesencephalon appears to be the seizure initiator.

C-FOS PROTOONCOGENE EXPRESSION

Among the immediate early genes, expression of the c-fos protooncogene has been proved to be a good candidate to reveal physiological activity as well as paroxysmal activity of neurones (27, 45). c-fos expression is induced during various types of epileptic syndromes in those regions of the brain directly responsible for the seizures (10, 27, 30). Interestingly, c-fos expression has been observed in the inferior colliculus in the mesencephalon of rodents during audiogenic GRE (27).

If our interpretation of the Fepi chimera results is correct, the brain localization of seizure-induced c-fos expression increases should match the structures implicated in audiogenic and photogenic GRE. The western blot technique was used to verify this hypothesis (36, 12). In mammals, visual and auditory activities increase c-fos expression in the corresponding sensory structures (13). Therefore, the first group of experiments examined the effect of visual and auditory epileptogenic stimulation in Fepi and Fhtz individuals. Since epileptic seizures are evoked only in the Fepi, the activity specific to seizures should be made obvious by this comparison. Samples from visual relay areas were selected for the experiment with ILS, and samples from acoustic relays areas were examined in the experiments with ISS.

Results are illustrated in Figure 5, showing that Fos and Fos-related antigen (FRA) protein expression is far more intense in Fepi than in Fhtz individuals in all the brain samples tested. This result indicates that, in the sensory relays, there is a higher activity of Fepi neurones with respect to Htz neurones. The experimental design did not allow us to distinguish hyperactivity induced in Fepi sensory relays by the triggering stimulus, from that of selfsustained generalized convulsions or from secondarily reverberated convulsions due to somatosensory activition which is likely to increase during motor seizures. In fact, we know that, in the absence of peripheral feedback from motor seizures (as obtained in the animals immobilized with flaxedil), the Fepi neurons show the bursting hyperactivity at rest and during sensory stimulation described above (14).

In a second group of experiments, only Fepi individuals were tested. In this case the western blot technique was applied to an identical set of brain structures in three animals in which photogenic-, audiogenic- or metrazole- (as a control) -induced seizures were obtained. This protocol allowed us to detect specific localizations of the increased protein expression according to the triggering stimulus used and therefore according to the type of seizure obtained. The results shown in Figure 6 are as

[2] The brain structures involved in the metrazole-induced seizures are more extensive then those involved in GRE, but the convulsions are the same.

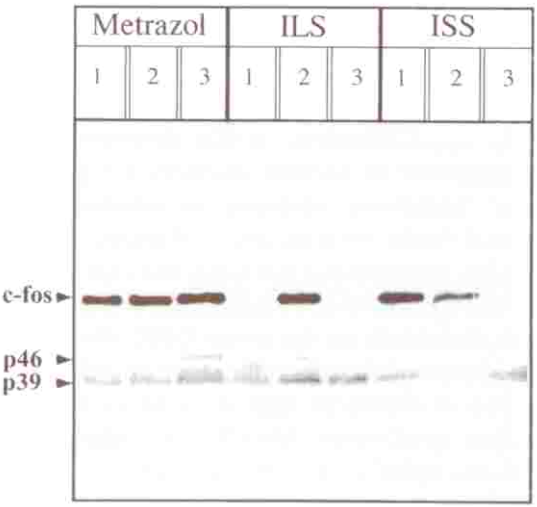


Fig. 6. - *Expression of c-fos after Metrazole-, ILS-, and ISS-induced seizures.*

Western blot obtained from three Fepi sacrificed 3 hours after seizures induced by Metrazole, or by audiogenic (ISS) or photogenic (ILS) stimulation. Samples were taken from the same structures in the three animals. 1: brain stem, 2: mesencephalon (tectum and tegmentum), 3: hippocampus. See text for explanations of the differential expression of c-fos in the three animals.

follows: a) all the structures tested display intense c-fos expression in the case of metrazole-induced seizures, which are known to involve the entire brain (25) [2]; b) intense c-fos expression is obtained only in the mesencephalon with light-induced seizures; c) with sound-induced seizures, intense c-fos expression is obtained, not only in the mesencephalon, but also in the bulbar region; d) FRAs are expressed to a lesser extent in all the samples studied, whatever the type of seizure obtained, therefore indicating a non specific effect [3].

Thus, metrazole-induced seizures are distinguished from the GRE. Photogenic GRE appears to have a localization distinct from audiogenic GRE, with only the latter showing an intense c-fos expression in the bulbar sample. This is interpreted as due to the sound-induced hyperactivity of the neurones of the primary sensory relays lying in the bulbar region, whereas the primary visual relays lie in the mesencephalon.

Finally, whatever the epileptogenic stimulus used, a common intense c-fos expression is observed in the mesencephalic samples which include the visual as well as the tecto-spinal relays. Therefore c-fos expression appears to be more intense in the animals having light-induced seizures than sound-induced seizures.

Altogether, the results obtained in the c-fos expression experiment are perfectly compatible with those obtained in the experiments using Fepi chimeras. They confirm that Fepi photogenic and audiogenic seizures have partially distinct localization. They also confirm once more the mesencephalic localization of the motor seizures initiator for Fepi GRE.

[3] Increase of FRA expression persists much longer (days) then Fos expression (hours) (40). Seizures were induced six hours before sacrificing the animal. This is the optimal time for the visualization of seizure-induced Fos expression. FRA expression has a much longer persistence time (40), and depends not only on the seizure history of the previous days but also on the net activity due to other uncontrollable factors during the previous days.

CONCLUSIONS

The present study shows that the Fepi is affected by at least two types of GRE, initiated by photogenic and by audiogenic stimulation. By constructing embryonic brain chimeras, it was demonstrated that both the GRE phenotypes can be transferred to normal chickens by grafting part of the Fepi neuroepithelium *in ovo*. Moreover, epileptic symptoms are specifically transferred, depending on which brain vesicles are exchanged. These results provide important information on the relationship between the still unknown genetic defect and the localization of the dysfunction to particular brain networks (4). Although one could argue that the dissection of the avian GRE obtained in the chimeras is somehow not representative of the situation seen in other species, we would argue that the various types of chimeras appear to be clear phenocopies of other spontaneous genetic reflex syndromes described in animal models and man (35). For example, our mesencephalic chimera appears to be a good phenocopy of the audiogenic reflex epileptic seizures of rodents when the triggering stimulus is the sound, and to the photomyologic response of man (39) when the triggering stimulus is the light. It is also similar to the human startling disease called startle epilepsy (2, 5). Finally, the movement-induced myoclonus of *Papio papio* (38, 28, 43), is also similar to the mesencephalic chimera although a movement-induced GRE has never been seen in unoperated Fepi individuals. These syndromes have in common the presence of paroxysmal motor symptoms in the absence of EEG paroxysms. However, syndromes without EEG paroxysms are considered "non epileptic" by many authors (34).

The above similarities between Fepi GRE, mesencephalic Fepi chimeras, and certain types of GRE seen in humans and other suggests a possible brain stem origin for GRE. Our results demonstrate that mesencephalic structures are involved in the initiation of Fepi paroxysmal motor symptoms. In other GRE systems, a brain stem origin has also been demonstrated for the initiation of rodent audiogenic GRE (27, 18), and for the initiation of *Papio papio* movement-induced myoclonus (29). This does not exclude the participation of other specific neural networks [for example, the sensory circuits corresponding to the triggering stimulus demonstrated in Fepi individuals using c-fos protooncogene expression (36, 12)]. Neither does this exclude the participation of other cortical structures. In particular, we have demonstrated that the Fepi prosencephalon is clearly involved in generating seizures in spite of the fact that, in birds, layered cortical structures are very poorly developed.

Over phylogenetic evolution, the prosencephalon appears to have acquired greater importance in *Papio papio* and man since they have higher degrees of corticalization than chickens. For example, focal ictal paroxysmal discharges can be evoked by ILS in the frontal cortex (38, 37) of the baboon and in the occipital cortex of man (39).

SUMMARY

The Fayoumi strain of chickens (Fepi) carries a recessive autosomal gene mutation in which homozygotes are afflicted with a photogenic and audiogenic reflex epilepsy.

Seizures consist of stimulus-locked motor symptoms followed by generalized self sustained convulsions. EEG recordings show spikes and spike and waves patterns at rest which are suppressed during seizures and replaced by a desynchronized pattern of activity. Neurones of the prosencephalon discharge in bursts at rest, while neurones of the mesencephalon are bursting during seizures.

Living neural chimeras were obtained by replacing specific embryonic brain vesicles in a normal chicken embryo with equivalent vesicles from a Fepi donor. These chimeras show that the epileptic phenotype can be totally or partially transferred from the Fepi to the normal chickens. Total transfer of photogenic and audiogenic seizures was obtained by substitution of both the prosencephalon and mesencephalon, while substitution of the prosencephalon alone resulted in transfer of interictal paroxysmal activity and substitution of the mesencephalon alone resulted principally in transfer of ictal motor symptoms.

Increased expression of the c-fos protooncogene, as revealed by the western blot technique, confirmed the distinct encephalic localizations of the symptoms of the photogenic and audiogenic reflex epilepsy of the Fepi shown with the methods of electrophysiology and brain chimeras. We conclude that the Fepi is a good model of brain stem reflex epilepsy and suggest that the brain stem is a generator of some other animal and human genetic reflex "epileptic syndromes".

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