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Effect of Hydrocortisone on the Phenotypic Expression and Inheritance of the *Fused* Gene in Mice

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Summary. This study was undertaken to examine the effects of hydrocortisone injected into male mice on the phenotypic expression and inheritance of the Fused (*Fu*) gene in their offspring. Data were obtained indicating that there is a hydrocortisone-susceptible period during spermatogenesis. Hydrocortisone injections of males during this period resulted in a statistically significant decrease in the proportion of phenotypically *Fu* offspring. Genetic analysis with the use of the closely linked recessive marker tufted (*tf*) demonstrated that the deficit of phenotypically *Fu* individuals among offspring is not caused by the differential death of gametes, zygotes or embryos. According to genetic data, this deficit is due to a decrease in the penetrance of the *Fu* gene and partly to its inherited inactivation. The possible mechanisms of the observed phenomenon are discussed.

Key words: Fused gene – Mice – Hydrocortisone – Gene inactivation

Introduction

In previous studies, we have demonstrated the interrelationship between events occurring in hormonal and morphogenetic systems (Naumenko et al. 1974; Belyaev et al. 1981 a). The domestication of animals, a process during which hormonal fluctuations played a crucial role in the reorganization of morphogenesis, provides a good example of this interrelationship (Belyaev 1979).

We have also shown that the variously penetrant mouse gene (*Fu*) (chromosome 17), which is known to produce fused shorter tails, can pass from an active to an inactive state (Belyaev et al. 1981 b). We have established that this passage is spontaneous, frequent, reversible, and inherited. The important findings were that all the F_1 hybrids from crosses between laboratory mice, homozygotes for the *Fu* gene, and wild *Mus bacterianus* were phenotypically normal (Reed 1937) and that the penetrance of the *Fu* gene was considerably decreased in the F_1 offspring from crosses between these homozygotes and wild *Mus musculus* (Belyaev et al. 1981 b).

Based on these findings and general considerations, we suggested that plasma concentrations of certain hormones, 11-hydroxycorticosteroids in particular, and the phenotypic expression of the *Fused* gene may be related.

The following experiments were undertaken to test this suggestion.

Materials and Methods

Corticosterone is the main plasma 11-hydroxycorticosteroid in mice. In the experiments, we utilized hydrocortisone, a hormone similar to corticosterone in chemical structure and physiological action.

The recessive marker *tufted* (*tf*), which is closely linked with the *Fused* (*Fu*) gene, was used in genetic analysis done according to the mating scheme previously described (Belyaev et al. 1981 b). To obtain males diheterozygous for the genes *Fu* and *tf*, the following cross was performed: $\frac{Fu+}{++}$ (CBA/lac-Fu) ♀♀ × $\frac{+tf}{+tf}$ (C57Bl/6J-*tf*) ♂♂.

Males with the phenotype *Fu*, non-*tf* and the genotype $\frac{Fu+}{+tf}$ were chosen from the F_1 obtained from this cross. Sexually mature $\frac{Fu+}{+tf}$ males were injected with 0.2 ml of saline and involved in the $\frac{+tf}{+tf}$ ♀♀ × $\frac{Fu+}{+tf}$ ♂♂ cross. Males were maintained with 4–5 females, as a rule, for 4–10 days and occasionally longer. Crosses in which males received saline before being penned with females served as controls.

Promptly after the control crosses, males were injected with 5 mg of hydrocortisone acetate (Gideon Richter, Hungary). The injected males were penned together with the $\frac{+tf}{+tf}$ (C57Bl/6J-*tf*) females. In some of the experiments, males were given a second injection of the same dosage 10–20 days

after the first one and then penned together with the genotypically $\frac{+tf}{+tf}$ females. The dosage of hydrocortisone was close to maximum. Its physiological effect was such that 25% of males failed to reproduce after treatment and 6–7% died.

The same mating scheme was used in the experimental series with the $\frac{+}{+}$ C57Bl/6J females.

The number of newborns with the *Fu* and normal phenotypes among offspring obtained from the control and experimental crosses was registered. Test of significance were done by using t- and F_φ-tests. In experiments undertaken to analyse the rate of the normalization of plasma 11-hydroxysteroids after the injections, males were given 2.5 mg of hydrocortisone acetate. Plasma 11-hydroxysteroids were measured by the standard method of acid fluorometry (Panov and Shalyapina 1968).

Results

To determine the period during which exogenous hydrocortisone was active, as well as its withdrawal rate, we measured the level of plasma 11-hydroxycorticosteroids in mice at various time points after the injections.

Thirty minutes after the injection, fluorescence intensity had increased to 119.7 ± 17.8 units, i.e., it was fourteenfold the baseline value. Because hydrocortisone contributes mainly to fluorescence intensity, its plasma concentration was 695.9 ± 111.6 μg/100 ml. Six hours after the injection, it had decreased to 23.0 ± 0.7, which corresponded to 60.0 ± 4.4 μg/100 ml of hydrocortisone. Twenty four hours later, fluorescence intensity had decreased to 10.7 ± 0.6 units, which significantly ($P > 0.95$) exceeded the control value (7.8 ± 0.6 units). It is difficult to determine the content of exogenous hydrocortisone and endogenous corticosterone because their relative concentrations are unknown. From the data obtained, it may be safely inferred that plasma 11-hydroxysteroids decreased during 24 h following hydrocortisone administration and that there were no differences in fluorescence intensity 72 h after it.

Temporal changes in the number of phenotypically *Fu* newborns from hydrocortisone treated fathers are shown in Fig. 1 and Table 1. The abscissa shows the number of days elapsed after treatment of males before birth of their offspring, and the ordinate shows the number of phenotypically *Fu* offspring expressed as a percentage of the expected number of genotypically *Fu* newborns. Taking a gestation period of 20 days, it was possible to reliably determine the time elapsed from the time point a male received hydrocortisone to the point he mated with a particular female.

The data of Fig. 1 are divided into 3 intervals from days 20–23, 24–25, and 26–32. There were no significant differences between the control and experimental crosses with a single hydrocortisone injection, when the values for these 3 intervals were summed (Fig. 1a, b). However, there was a significant decrease in the proportion of the phenotypically *Fu* individuals ($F_\varphi = 4.23$, $P > 0.95$) in litters born 24–25 days after the treatment of males with hydrocortisone. Consequently, a single injection of hydrocortisone produces a significant decrease in the proportion of phenotypically *Fu* offspring when 4–5 days have elapsed from hydrocortisone treatment to mating.

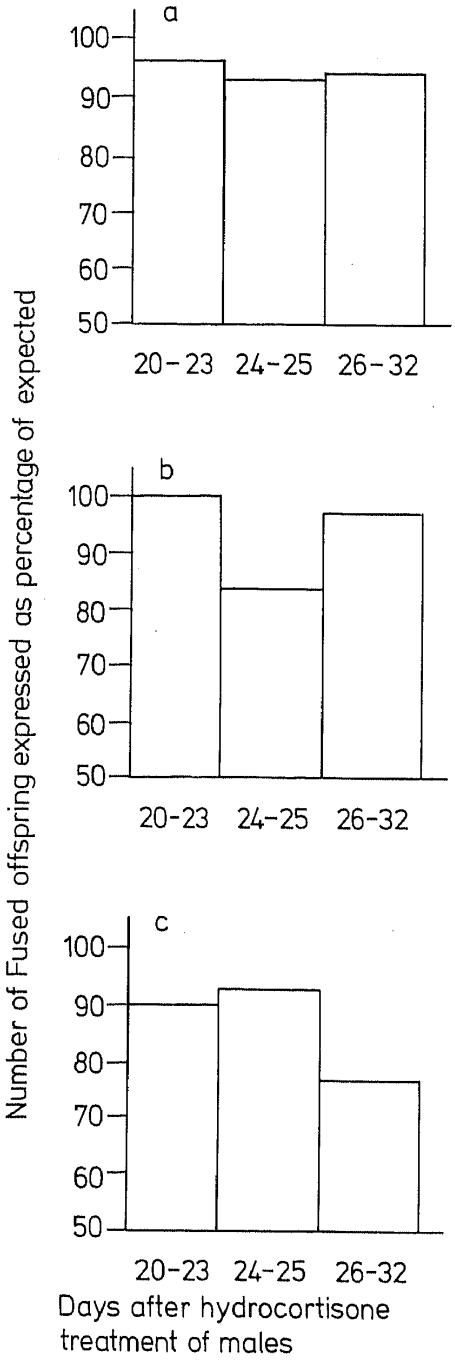

Fig. 1a–c. The number of phenotypically *Fused* offspring in the $\frac{+tf}{+tf}\varphi\varphi \times \frac{Fu+}{+tf}\delta\delta$ crosses expressed as a percentage of expected. **a** Males received saline before the mating experiments. **b** Males received a single hydrocortisone injection. The difference is significant at $P > 0.95$ by the F_φ-test at days 24–25. **c** Males received multiple injections of hydrocortisone. The difference is significant at days 26–32 ($P > 0.99$)

Table 1. The F_1 segregation ratio in the $\frac{+\ tf}{+\ tf}$♀♀ × $\frac{Fu+}{+\ tf}$♂♂ cross

Experimental conditions / Days from injection of hydrocortisone into males to birth of F_1 offspring	20 – 23 days		24 – 25 days		26 – 32 days		Total
	Fu	+	*Fu*	+	*Fu*	+	
Control (saline injection into males)	102	112	119	138	75	84	630
Single hydrocortisone injection into males	134	124	110	150	104	106	728
Multiple hydrocortisone injections into males	89	109	104	122	94	148	666

Table 2. Results of the phenotypic identification of offspring from the $\frac{+\ tf}{+\ tf}$♀♀ × $\frac{Fu+}{+\ tf}$♂♂ crosses in which males received either saline or hydrocortisone

Experimental conditions	Offspring phenotype				*Fu* non-manifestation %
	Fu, non-*tf*	non-*Fu*, *tf*	non-*Fu*, non-*tf*	*Fu*, *tf*	
Control (saline)	90	111	3	–	3.2
Experiments (hydrocortisone)	188	212	22	–	10.5*

* The difference in the percentage of the phenotypic non-manifestation of the *Fused* gene is significant at $P > 0.95$ by the F_φ-test

In a part of the experiments, males received multiple (2 or 3) injections of hydrocortisone with an interval of 10–20 days after each. The data of these experiments are plotted in Fig. 1c. In the offspring from treated fathers, the penetrance of the *Fu* gene was significantly ($F_\varphi = 9.16$, $P > 0.99$) lower than in the controls. There was also a period when the relative proportion of phenotypically *Fu* offspring was significantly smaller than that in the control experiments. This hormone-susceptible period was shifted to days 26–32 after the last treatment. The deficit of the phenotypically *Fu* individuals was the largest 27–28 days after the treatment, attaining 72% of the expected number (the ratio of the phenotypically *Fu* individuals to the normal ones was 48 : 85). It may be concluded that the period most susceptible to hydrocortisone is 7–8 days before fertilization in the experiments in which it was given more than once. The shift of the susceptible period was the presumable consequence of hydrocortisone pretreatment.

Three observations emerged from comparisons of the experiments with single and multiple injections. Firstly, whether given in a single dosage or not, hydrocortisone produced a significant decrease in the number of phenotypically *Fu* offspring. Secondly, the multiple injections were more efficient as judged by the significant ($P > 0.99$) decrease in the proportion of *Fu* offspring compared to mice that had received a single injection of hydrocortisone. Thirdly, there was a shift of the period of high susceptibility of maturing spermatozoa to hydrocortisone from 4–5 days to 7–8 days before fertilization. What was the cause of the lack of phenotypically *Fu* individuals among the offspring of hydrocortisone-treated fathers? The differential viability of zygotes, gametes, embryos or, perhaps, the non-manifestation of the *Fu* gene in some of the heterozygous offspring?

The closely linked recessive marker tufted (*tf*) allowed us to test both possibilities. Identification of the trait *tf* is usually carried out in mice older than a month. When the control and experimental data were compared, no significant differences were found between the segregation pattern for *tf* in offspring. As can be seen in Table 2, the ratios of the alternative classes for the controls (93 non-*tf*: 111 *tf*) and for the treated mice (210 non-*tf*: 212 *tf*) did not deviate from the expected 1 : 1. There were also no differences at the susceptible time points. The ratio was 37 non-*tf*: 36 *tf* at days 24–25 among offspring of fathers that had received single hydrocortisone injections, and it was 29 non-*tf*: 33 *tf* at days 26–32 among offspring of fathers that had received multiple ones. In contrast, the percentage of *Fused* offspring at the time points examined was significantly smaller in the experimental series. This refuted the possibility that the deficit of the phenotypically *Fused* individuals at the susceptible periods was due to the differential viability of gametes, zygotes or embryos. We could say with certainty that the deficit was caused by non-manifestation of the *Fu* gene.

Twenty-two phenotypically non-*Fu*, non-*tf* mice lived to sexual maturity. Of these, 19 produced off-

spring. The fathers of 5 had received a single injection of hydrocortisone, the fathers of 14 had received multiple ones. What was the genotype of these individuals with respect to the *Fu* gene? Did there occur an inherited inactivation of the *Fu* gene like the one we had previously observed (Belyaev et al. 1981 b)?

Based on the data of genetic analysis, the mice were divided into two groups (Table 3). Group 1 consisted of 6 mice. Among the 450 F_1 and F_2 offspring of these mice none were phenotypically *Fused*, but the segregation ratio for *tf* was 42 non-*tf*: 36 *tf*, not significantly deviating from the 1 : 1 expected. Group 2 was composed of 13 phenotypically non-*Fu*, non-*tf*, all of which produced phenotypically *Fu* offspring in the F_1. However the total segregation ratio of 43 *Fu*: 87 phenotypically normal individuals significantly deviated from the one expected ($\chi^2 = 14.9$, $P > 0.99$). In this case, the penetrance of the *Fu* gene was only 66% compared to 94% in the controls. The ratio for *tf* phenotype, 14 phenotypically normal individuals: 16 *tf*, ruled out differential death of gametes, zygotes or embryos as the alleged cause of the deficit of *Fu* individuals. Consequently, the data indicated that the penetrance of the *Fu* gene was sharply decreased in the offspring of at least some of the mice of Group 2.

Three phenotypically non-*Fu*, non-*tf* individuals were identified in the control crosses (Table 2). When testcrossed with the $\frac{+\,tf}{+\,tf}$ mice, all 3 yielded phenotypically *Fu* offspring with a total segregation ratio of 11 *Fu*: 13 normal, thereby proving that their genotype was $\frac{Fu+}{+\,tf}$.

Comparisons of the overall control and experimental data demonstrated a significant increase in the relative proportions of phenotypically non-*Fu*, non-*tf* mice (Table 2) and also of mice having the *Fused* gene in inactive state among them (Table 3). Perhaps some of the 6 mice which gave no *Fused* offspring had a recombinant chromosome giving rise to the genotype $\frac{++}{+\,tf}$? This appeared unlikely because there was no other class of recombinants with the genotype $\frac{Futf}{+\,tf}$ in the experiments. What appeared plausible was a *Fu*→[*Fu*] event, i.e., an inherited switching off of the *Fused* gene. If so, the genotype of these mice is $\frac{[Fu]+}{+\;\;tf}$ (Belyaev et al. 1981 b). The distribution of phenotypically normal newborns (non-*Fu*, non-*tf*) indicates that the great majority (20 of 22) of mice were born at a time when the penetrance of the *Fu* gene was decreased.

In the subsequent experiments, males with the genotype $\frac{Fu}{+}$, which had received a single dose of hydrocortisone, were crossed with the C57Bl/6J females. These females differ from the *tf*/*tf* (C57Bl/6J-*tf*) females in the penetrance of the *Fu* gene in offspring. It was significantly lower in the former (72.1%, $F_\varphi = 43.8$, $P > 0.99$) than in the latter (93.9%). These differences are shown in Tables 1; 4 and Figs. 1; 2. The two sublines also significantly differed in the values for plasma 11-hydroxycorticosteroids, which were 17.6 ± 0.4 µg/100 ml in $\frac{+}{+}$ females and 8.3 ± 0.6 µg/100 ml in the $\frac{tf}{tf}$ females ($t_d = 12.9$, $P > 0.99$). The difference in the number of the phenotypically *Fu* offspring attains statistical significance on days 20–23 ($F_\varphi = 6.94$, $P > 0.99$) (Fig. 2). The penetrance of the *Fu* gene in mice born 22–23 days after treatment of the fathers, i.e., when the maturing spermatozoa were hydrocortisone-susceptible, was as low as 50%, and the segregation ratio was 30 *Fu*: 87 normal. In this case, the spermatozoa were at the hydrocortisone-susceptible period 2–3 days before they fertilized the eggs. The question was, why was the hydrocortisone-susceptible period shifted to 2–3 days before fertilization in this case, whereas it occurred 4–5 days before it in the cross of these males with the *tf*/*tf* (C57Bl/6J-*tf*) females? The results of the analysis of the $\frac{+}{+}$ C57B1/6J♀♀ × $\frac{Fu}{+}$ ♂♂ cross was as follows.

Table 3. Genetic analyisis of the F_1 phenotypically normal (non-*Fu*, non-*tf*) offspring from the $\frac{+\,tf}{+\,tf}$♀♀ × $\frac{Fu+}{+\,tf}$♂♂ crosses in which males received hydrocortisone

Expected genotype of the phenotypically normal F_1 offspring	No. mice test-crossed	Phenotypes of offspring					
		$F_1 \times \frac{+\,tf}{+\,tf}$[a]				$F_2 \times \frac{+\,tf}{+\,tf}$; $F_2 \times F_2$	
		Fu	Normal	*tf*	non-*tf*	*Fu*	Normal
$\frac{[Fu]+}{+\,tf}$	6	0	178	37	42	0	272
$\frac{Fu+}{+\,tf}$	13	43	87	16	14	–	–

[a] Only some of the F_1 individuals were anlayzed for the trait *tufted*

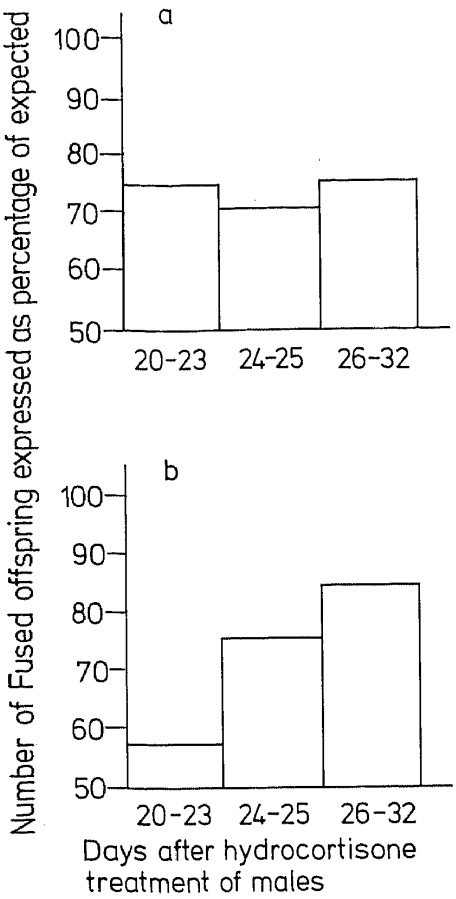

Fig. 2a and b. The number of phenotypically *Fused* offspring in the $\frac{+}{+}\,♀♀ \times \frac{Fu}{+}\,♂♂$ crosses expressed as a percentage of expected. **a** Males received saline before the mating experiments. **b** Males received a single hydrocortisone injection. The difference is significant at $P > 0.99$ by the F_φ-test at days 20–23

During the first 3 days, when males and females were maintained together, 70% of the treated males fertilized minimally one female; this means that in all the following matings the females were fertilized with sperm modified by exogenous hydrocortisone. The situation was different in the $\frac{+tf}{+tf}♀♀ \times \frac{Fu+}{+tf}\,♂♂$ cross. During the first 3 days of joint maintenance, only 23% of the males fertilized females and, for this reason, the majority of treated males fertilized females for the first time with mixed sperm, a portion of which was modified by hydrocortisone and the other not. Because of this different time course of mating in the two sublines, the susceptible period was possibly shifted in the experiments with single hydrocortisone administration. The shift may also be accounted for by the shorter gestation period of the +/+(C57Bl/6J) females.

Discussion

Exogenous hydrocortisone produces changes in the template activity and chemical structure of chromatin (Argutinskaya et al. 1973), and it increases mutation rate during spermatogenesis in mice (Loginova and Kerkis 1975).

The aim of the present work was to determine whether or not there is a relation between plasma 11-hydroxicorticosteroid level in parents and the phenotypic expression of genes in offspring. Since the *Fused* gene causes a conspicuous phenotype, we used it as an advantageous system for testing the effects of exogenous hydrocortisone injected into males.

Our results demonstrated the efficiency of hydrocortisone injections. The percentage of phenotypically *Fu* offspring of fathers that had received hydrocortisone decreased. Genetic analysis indicated that this decrease is the result of the lower penetrance of the *Fu* gene and partly of its inherited inactivation.

The only conceivable mechanism transmitting the signal of modified hormone level from father to offspring is altered structure or function of spermatozoa. Changes in some of the properties of chromatin may be, in all probability, responsible for the phenomenon observed. This appears plausible when one recalls the structural characteristics of the chromatin of maturing spermatozoa. The obvious question is, what is the biological significance of this hormone susceptible period of spermatogenesis? In mice, the duration of spermatogenesis is 34 days, on the average, and meiosis commences 26 days before the maturation of spermatozoa. Spermiogenesis occurs during the last 13 days. It is then that the structural and functional changes in the cells are associated with nuclear elongation, repatterning of chromosome structures and loss of

Table 4. The segregation ratio in the $\frac{+}{+}♀♀ \times \frac{Fu}{+}\,♂♂$ cross

Experimental conditions \ Days from injection of hydrocortisone into males to birth of F_1 offspring	20 – 23 days		24 – 25 days		26 – 32 days		Total
	Fu	+	*Fu*	+	*Fu*	+	
Control (saline injection into males)	80	139	42	78	11	19	369
Single hydrocortisone injection into males	74	186	16	27	45	62	410

cytoplasm. In rats, for example, this is the period most vulnerable to the effects of hypophysectomy (Austin and Short 1972). Biochemical studies of the terminal stages of spermatogenesis indicate that 4–8 days before the complete maturation of spermatozoa and their traverse through the epididymus, the spermatidal basic nuclear proteins are replaced by protamines and chromatin packing in the spermatozoa is brought to completion (Goldberg et al. 1977). Thus this important period of spermatogenesis may not by chance be susceptible to hydrocortisone in the experiments performed.

A population of cells involved in spermatogenesis is naturally heterogenous and so is its sensitivity to an exogenous hormone and, hence, this hormone differently affects each stage of spermatogenesis. Consequently in our experiments, only those cells which were at the susceptible phase during the first two days after hydrocortisone treatment were modified.

The variability of the expression and inheritance of the *Fu* gene under the effect of exogenous hydrocortisone are not chance events, inasmuch as high spontaneous variability is characteristic of this gene. In contrast, *tf,* a gene stable in expression and a 100% penetrant, was found to be insensitive to the hormone (Tables 2 and 3). Clearly, not all the genes behave like *Fu* in response to an exogenous hormone. On the other hand, the *Fused* gene is not an exception. After we had completed the experiments described above, the gene affecting mouse susceptibility to cortisone-induced cleft palate was tentatively located on chromosome 17 distal of the *Fu* gene (Gasser et al. 1981). It is pertinent to note that the *Fu* gene is within the region of the t-haplotypes, i.e., it is in the region where the loci responsible for many genetic abnormalities are (Bennett 1975). Drastic changes in the chromatin properties of the elongated regions of chromosome 17 seem to be the underlying mechanisms of these genetic abnormalities (Lyon et al. 1979). With all this in view, an attractive assumption appears to be the dependence of the penetrance of the *Fu* gene and its inherited inactivation on the state of chromatin in the region of chromosome 17. There is cytogenetic evidence for chromatin state being inherited during sexual reproduction (John and Gabor-Miklos 1979).

In this context, it would be appropriate to comment upon the recombinational distance between the genes *Fu* and *tf.* Estimates of this distance were based on the appearance of the recombinant chromosome *Futf.* Although 292 phenotypically *Fu* offspring obtained in the experimental and control crosses were screened for tufted, not a single recombinant was identified. In our previous study, where diheterozygotes for the *Fu* and *tf* were females, the recombination distance between the two genes was estimated as 1 cM which is in agreement with the data in the literature (Dunn et al. 1962). This may be, with more certitude, accounted for by the very much lower crossing over frequency in this region of chromosome 17 in males (Dunn and Bennett 1967). Taken together, all these observations make unlikely the explanation that the appearance of 6 phenotypically non-*Fu,* non-*tf* mice, which produced normal offspring only in the testcrosses, was the consequence of a recombination between the genes *Fu* and *tf.* The absence of phenotypically *Futf* individuals among 204 offspring in the control matings also argues against this explanation also (Table 2).

The results of this study also raised the question of the effects of the level of plasma 11-hydroxicorticosteroids in females on the manifestation of the *Fu* gene in offspring. Reed's experiments (1937) and subsequently ours (Belyaev et al. 1981 b), demonstrated that there occurs a drastic decrease in the penetrance of the *Fu* gene to its 100% non-manifestation in offspring from crosses between *Fu* homozygous males and wild *Mus bactrianus* and *Mus musculus* females. Measurements of plasma 11-hydroxicorticosteroid concentrations in wild females established much elevated values compared to those in strain mice. It thus seems probable that not only males, but also females, conform to the general pattern: a increase in plasma 11-hydroxicorticosteroids (endogenous or exogenous) produces a decrease in the penetrance of the *Fu* gene and occasionally its inherited inactivation in offspring.

Profound reorganization of the hormonal system is a widespread evolutionary process. This was borne out of our previous study (Belyaev 1979). The results of this study further support the suggestion that hormonal changes taking place in animals subjected to intense artificial selection during domestication is a strong inducer of extensive and explosive variability inherent in domestication. For this reason, selection, by affecting along with others hormonal regulatory mechanisms, provokes developmental destabilization. It sharply accelerates the emergence of new forms of animals and thus becomes destabilizing.

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