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Strain and Age Effects on Immobilization Stress in JAX Rabbits (41088)[1]

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Abstract. A brief immobilization stimulus was used in surveying the pituitary adrenal responsiveness of 12 strains of JAX rabbits. Healthy male adult rabbits of 7.3 to 25.3 months in age of the following strains were used: IIIVO/J, III/J, IIIEP/J, IIIC/J, WH/J, X/J, AC/J, ACEP/J, AX/J, AXBU/J, B/J, and OS/J. Blood samples were obtained daily from the marginal veins of the ears of each rabbit at 0, 10, 16, and 22 min postimmobilization for 5 consecutive days and assayed for corticosterone and cortisol by radioimmunoassay. It was observed that the average steroid level (by strain) is highest in strain III/J and lowest in strains IIIVO/J and OS/J. In regard to the changes in steroid level occurring during immobilization, at 0 min time corticosterone levels in strain X/J are significantly higher than the levels in the other 11 strains, but at 10, 16, and 22 min strain III/J corticosterone levels are significantly higher. The elevation in plasma corticosterone at 10 min postimmobilization is significant in all strains of rabbits but the response tends to plateau within 16 min. In regard to the secretion of cortisol, no significant change in the plasma level of cortisol was elicited by short periods of immobilization on 5 consecutive days. Following the survey of immobilization stress responses in 12 rabbit strains, 4 strains (X, III, AC, IIIVO) were selected to study the effect of age on this response. Three age groups were employed: 55 to 60, 100 to 109, and 318 to 412 average days age. It was observed that there are age-related increases in the response to immobilization, significant in strains III and X, and in the response to exogenous ACTH, significant in strain X.

Although the rabbit was employed in an early classic experiment dealing with the question of neural control of the pituitary adrenal system (1), more recently the rabbit has not been widely used for this purpose. In view of the success of this early experiment, there is reason to believe that the rabbit is a suitable subject for experimentation on the mammalian pituitary adrenal system; but one in which there is a relative absence of modern assay data. In preliminary experiments on the rabbit, it was found that there are significant differences in the corticosterone responses to ketamine HCl in various strains of rabbits (2). On the basis of this information, a decision was made to survey the pituitary–adrenal activity in a large number of inbred and partially inbred strains in response to immobilization stress. Since there is great genetic homogeneity within each rabbit strain, we believed a survey of the major trends in these responses could be accomplished with a minimum number of rabbits per strain. Comparison of the corticosteroid responses, among the strains, in this initial experiment led to a second experiment in which the responses to immobilization stress and to exogenous ACTH were compared in 4 of the initial 12 strains as a function of age.

Materials and methods. *Survey experiment.* The rabbits were healthy male adults from the inbred and partially inbred strains maintained at the Jackson Laboratory. They were fed commercial laboratory chow (Purina Rabbit Chow Checkers, 20% protein) with water *ad libitum*. In view of the high degree of genetic homogeneity of the rabbits used, three males were chosen from each of 12 strains. Strain designations and average ages in months of the subjects used are as follows: IIIVO/J (16.3 ± 2.4), III/J

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(25.3 ± 0.3), IIIEP/J (9.3 ± 0.7), IIIC/J (14.0 ± 1.0), WH/J (11.7 ± 1.3), X/J (7.3 ± 0.7), AC/J (18.3 ± 0.3), ACEP/J (10.3 ± 0.9), AX/J (9.3 ± 0.9), AXBU/J (8.3 ± 0.3), B/J (23.3 ± 2.7), and OS/J (9.0 ± 2.1) (4, 5, 6, 7). These have been abbreviated in the text to: IIIVO, III, IIIEP, IIIC, WH, X, AC, ACEP, AX, AXBU, B, and OS, respectively. Blood samples (50 μl) were taken between 0800 and 1330 hr from the marginal ear veins and collected in heparinized microhematocrit tubes. This restricted sampling period was adapted since a circadian rhythm has been shown for some other parameters in the rabbit (8–10). Illumination of the rabbit pens was daylight from approximately 0530 to 2030 hr. The order of sampling from each strain was randomized daily to minimize any effect due to sampling sequence. Samples were taken between 7/26/76 and 8/14/76 and were processed by radioimmunoassay in the following 4 months. Rabbits remained in their home cages until just prior to sampling when they were immobilized by wrapping in a burlap cloth which was secured with towel clamps as previously reported (11). Samples were taken at 0, 10, 16, and 22 min after immobilization. Samples were centrifuged within 30 min of collection. The plasma was placed in phosphate buffer and stored frozen at −20° until assayed for corticosterone and cortisol.

Strain and age experiment. Fifteen healthy, male rabbits from each of the following four strains were chosen: X/J, III/J, IIIVO/J, and AC/J. In each strain, the animals were divided into three age groups of five rabbits each, whose average ages ranged from 55 to 60 days, from 100 to 109 days, and from 318 to 412 days. Blood samples were collected at 0 and 22 min after immobilization under conditions identical to those described for the Survey Experiment. Within 5 days of the immobilization stress experiments, samples were collected at 0 and 180 min after saline and at 180 min after ACTH (Acthar Gel, Armour 1 U/kg, i.m.). In these tests, sampling time was shortened by the employment of a team of three people in the restraining and bleeding procedure so that blood sampling was completed at approximately 15 sec following removal from the cage.

Assays for corticosterone and cortisol. Plasma concentration of corticosterone (total) was measured by a radioimmunoassay previously described for cortisol (12), but here it was modified to measure corticosterone by employing corticosterone standards. Tritiated cortisol (New England Nuclear) and an antiserum specific for corticosterone and cortisol (Endocrine Sciences, F21-53) were employed. Plasma samples from individual rabbits were assayed in triplicate in 25 separate assays. The assay was sufficiently sensitive to detect 0.1 ng corticosterone. The accuracy of the radioimmunoassay was estimated by recovering quantities of corticosterone added to adrenalectomized rat plasma. When 10 μg/dl was added, 11.7 ± 2.3 μg/dl (5) was recovered and when 20 μg/dl was added, 19.6 ± 1.9 μg/dl (5) was recovered. Pools of plasma were repeatedly assayed to estimate the precision of the assay. The mean concentration of corticosterone, μg/dl ± SE (interassay coefficient of variation), was 7.72 ± 0.40 μg/dl (5.1%) in 10 repetitions.

To assay rabbit plasma levels for cortisol, cortisol standards and a highly specific antiserum for cortisol (BIO RIA, AS-COR NO. 143) was substituted in the radioimmunoassay procedure described above. Cross-reactivity to corticosterone is reported to be less than 1%.

Statistical analysis. The statistical analysis of the experimental data is based upon an analysis of variance model for repeated measurements in the same subject. In the application of this model, the observations were subjected to a square-root transformation to achieve stability of variation within the various experimental classes. The values presented in Tables II to IV were obtained by squaring the transformed values. Subsequent to the analysis of variance, and dictated by the significant interactions found there, *a posteriori* methods for the paired comparison of means (Student–Newman, Keuls test) were employed. Statistical significance was defined as $P < 0.05$ (13).

Results. In the survey experiment, each rabbit was immobilized for a period of 22 min on 5 consecutive days and the plasma was sampled after 0, 10, 16, and 22 min of immobilization. The data from Days 1 through 5 were subjected to analysis of variance (Table I). It was found that the differences among the strains, the days of sampling, and the times of sampling are significant. When the strains are ranked in order of mean corticosterone concentrations (Table II), it is found that the mean steroid level in strain III is the highest and in strains IIIVO and OS, the lowest. When the mean corticosterone levels for Days 1 through 5 are ranked, the mean level on Day 4 is less than the levels of the other 4 days and the levels on Days 1, 2, 3, and 5 are not significantly different.

In regard to the comparison of strains within each sampling time, in Table III it is shown that at 0 min sampling time, the plasma corticosterone level in strain X is significantly higher than in the other 11 strains, while in strains IIIC and IIIVO, it is significantly lower. When the corticosterone response to immobilization occurs, a significantly larger response appears in strain III at 10, 16, and 22 min postimmobilization, while the steroid response of strain X is relatively small. The response of strain IIIC is ranked next to strain III while the response of strain IIIVO is among the least.

In Table IV, it can be seen that the surge in plasma levels in corticosterone during the first 10 min of immobilization is highly significant in all strains of rabbits. However, in strains X and OS, this surge tends to plateau after 10 min while in 7 of the remaining 10 strains, it plateaus after 16 min. In only 3 strains, IIIC, ACEP, and IIIVO, does the rise continue to be significant beyond 16 min.

In the initial survey of the immobilization response in 12 strains of rabbits, the average age of several groups deviated significantly from 12 months. Since age is also a factor in determining the stress response, the age variable was investigated in four of these strains (Table V). Comparison of three age groups in these four strains revealed significant age related trends in the responses to immobilization and to exogenous ACTH. The 22-min period of immobilization evokes significant steroid responses in each age group of the four strains. The 0-min level from which this response originated is not significantly different among the four strains or the three age groups. The 22-min postimmobilization response is significantly greater in strains III and X than in strains AC and IIIVO and significantly greater in the 318- to 412-day

TABLE I. ANALYSIS OF VARIANCE OF MEAN CORTICOSTERONE CONCENTRATIONS (VALUE TRANSFORMED TO SQUARE ROOT) ON DAYS 1–5 FOR THE 12 RABBIT STRAINS

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F*
Among rabbits				
Strains	11	122.29	11.11	6.49* = $F(11, 24)$
Error	24	41.06	1.71	
Within rabbits				
Days	4	13.74	3.43	6.59* = $F(4, 96)$
Days × Strains	44	22.91	0.52	0.998 NS = $F(44, 96)$
Error	96	50.06	0.52	
Time	3	666.81	222.27	743.64* = $F(3, 72)$
Time × Strains	33	59.80	1.81	6.06* = $F(33, 72)$
Error	72	21.52	0.29	
Days × Time	12	3.79	0.31	2.80* = $F(12, 288)$
Days × Time × Strains	132	11.67	0.08	0.78 NS = $F(132, 288)$
Error	288	32.57	0.11	

* Indicates statistical significance at the 0.01 level. NS indicates no statistical significance at the 0.01 level.

TABLE II. RANK ORDER OF MEAN CORTICOSTERONE LEVELS (μg/dl) BY RABBIT-STRAIN FOR 5 DAYS (N = 60)

III	IIIC	AC	AXBU	IIIEP	WH	AX	B	X	ACEP	IIIVO	OS
14.81	10.24	10.20	10.06	9.86	9.83	9.56	8.84	8.18	5.85	5.26	5.11

Note. Underlined values are not significantly different, $P > 0.05$.

age groups. The age effect on the immobilization response is especially large in strain III.

In the injection experiments if the effect of strain is examined without regard to age, strain X exhibited significantly greater steroid levels at 0 and 180 min after saline and at 180 min after exogenous ACTH. In this regard, it should be noted that the latency from removal from the cage to completion of blood collection is so short, about 15 sec, that the 0-min corticosteroid levels are true values unaffected by handling stress. The effect of exogenous ACTH is greater in strain X not only on the basis of the magnitude of the 180-min post-ACTH steroid level but also relative to the 180-min postsaline level. In regard to the age groups, the differences at 0 and 180 min after saline are negligible, but at 180 min after exogenous ACTH, there is a very significant increase in the ACTH evoked steroid response in the 318- to 412-day age group.

In regard to cortisol levels, an estimate of the concentrations of cortisol in plasma samples collected on Days 1 and 6 of the survey experiment was made in a separate radioimmunoassay. The mean estimated cortisol concentration at 22 min postimmobilization in the 12 strains of rabbits on Day 1 is 1.03 ± 0.12 and on Day 6 it is 1.11 ± 0.09 μg/dl.

Discussion. The data collected in this paper on the responsiveness of the rabbit pituitary adrenal system to the stress of a brief period of immobilization increase our ability to discriminate in the selection of a suitable strain of rabbit for a given experimental goal. Different strains exhibit different response patterns. When the interaction between strains and sampling time is examined by comparing the corticosterone levels achieved by the 12 strains at 22 min of immobilization: three groups of rabbit strains may be discerned: high, low, and average achievers. Strain III achieves a high corticosterone level; strains ACEP, IIIVO, and OS, a low level and strains AC, IIIEP, AXBU, WH, AX, and B, an average level. Strains IIIC and X appear to be situated at intermediate levels between these three groups.

Consideration of the initial as well as final levels of corticosterone identifies other groups of rabbits within the 12 strains. The best estimate of resting corticosterone levels in the 12 strains of rabbits in the morning hours is the concentration measured at 0 min. This level ranges from 0.41 to 2.91 μg/dl with strain X exhibiting a value significantly greater than the 11 other strains and strains IIIVO and IIIC exhibiting levels significantly lower than the remaining 10 strains.

Another consideration which arises when viewing these results is that the average age of the subjects in the 12 strains of the survey experiment ranges from 7 to 25 months. In 3 of the strains (III, IIIC, AC) in which the average age was 25, 14, and 18 months, steroid levels are highest at 22 min. On the other hand, in 2 other strains (B, IIIVO) in which the average age was 23 and 26 months, the 22-min levels are low. To observe the effect of age on the immobilization response more completely, 4 of the 12 strains (III, IIIVO, AC, X) were selected for a second experiment. In the survey experiment, these 4 selected strains displayed distinctive patterns of immobilization evoked corticosterone responses: strain III displays low initial and high final corticosterone levels while in strain IIIVO, there are low initial and low final levels and in strains AC and X, there are high initial and intermediate final levels. In the age experiment, strain III again displays the highest

TABLE III. RANK ORDER OF PLASMA CORTICOSTERONE LEVELS (μg/dl) BY RABBIT STRAINS FOR THE FOUR SAMPLING TIMES ($N = 15$)

Time	Strains											
Time 0′	X	B	AC	WH	AX	ACEP	AXBU	OS	III	IIIEP	IIIVO	IIIC
	2.95	2.43	2.42	2.29	2.25	1.35	1.22	1.06	0.83	0.81	0.48	0.41
Time 10′	III	AXBU	WH	AX	AC	B	IIIEP	IIIC	X	OS	ACEP	IIIVO
	13.46	9.30	9.18	8.88	8.44	8.41	8.07	8.01	7.60	4.58	4.31	2.57
Time 16′	III	IIIC	IIIEP	AC	WH	AXBU	AX	B	X	ACEP	OS	IIIVO
	20.26	13.80	13.32	13.18	12.89	12.82	12.18	10.96	9.73	6.92	6.35	6.22
Time 22′	III	IIIC	AC	IIIEP	AXBU	WH	AX	B	X	ACEP	IIIVO	OS
	23.62	17.72	16.07	15.97	15.76	14.29	14.06	12.53	11.29	9.73	9.24	7.73

Note. Underlined values are not significantly different, $P > 0.05$.

TABLE IV. PLASMA CORTICOSTERONE LEVELS (μg/dl) BY SAMPLING TIMES FOR EACH RABBIT STRAIN ($N = 15$)

Strain	Sampling time in minutes 0′	10′	16′	22′
III	.83	13.46	20.26	23.62
IIIC	.41	8.01	13.80	17.72
AC	2.42	8.44	13.18	16.07
AXBU	1.22	9.30	12.82	15.76
IIIEP	.81	8.07	13.32	15.97
WH	2.29	9.18	12.89	14.29
AX	2.25	8.88	12.18	14.06
B	2.43	8.41	10.96	12.53
X	2.95	7.60	9.73	11.29
ACEP	1.35	4.31	6.92	9.73
IIIVO	.48	2.57	6.22	9.24
OS	1.06	4.58	6.35	7.73

Note. Underlined values are not significantly different, $P > 0.05$.

postimmobilization response while strain IIIVO is the lowest and strains AC and X are intermediate. When the age of strains X and AC is comparable, the immobilization response of strain X exceeds that of strain AC. Furthermore, there is a notable increase in the immobilization response in the 318- to 412-day age group. In regard to the response to exogenous ACTH, there is an even more pronounced increase in the response in the 318- to 412-day age group. In the 55- to 60- and 100- to 109-day age groups, the response to ACTH tends to be less than the response to immobilization while in the 318- to 412-day age group, the response tends to be greater. This suggests that there are separate rates of maturation of these two aspects of pituitary adrenal function. Finally, it is interesting to note that although rabbit strains III and IIIVO are closely related genetically, the pituitary adrenal systems differ in their performance.

Another aspect of this experiment is related to the well-documented finding that chronic stimulation of the rabbit adrenal gland with large doses of porcine ACTH results in a radical alteration in the steroid composition of the adrenocortical secretion (14–17). Normally the ratio of corticosterone to cortisol in adrenal venous blood is about 20:1. After chronic treatment with ACTH this ratio approaches unity. In view of the repetitive daily immobilization stress to which the rabbits in this experiment were subjected, albeit for only 6 days, cortisol levels were also monitored. However, since the cortisol levels after 22 min of immobilization on Days 1 and 6 are not significantly different, we have no evidence of a shift in adrenocortical secretion from cor-

TABLE V. EFFECT OF AGE ON CORTICOSTEROID RESPONSE IN FOUR RABBIT STRAINS[a]

Strain	Age (days ± S.E.)	Immobilization 0′	Immobilization 22′	Saline 0′	Saline 180′	ACTH (1 IU/kg, im)
IIIVO	56 ± 0	2.6 ± 2.2	10.3 ± 6.8	0.9 ± 0.9	1.9 ± 1.3	4.2 ± 2.4
	109 ± 4	1.9 ± 1.9	10.3 ± 4.5	1.2 ± 0.8	2.4 ± 0.9	5.9 ± 4.4
	369 ± 19	2.0 ± 0.6	15.8 ± 6.6	0.6 ± 0.3	1.9 ± 0.8	20.3 ± 2.9
X	60 ± 1	3.7 ± 2.1	17.0 ± 6.8	2.5 ± 3.3	7.0 ± 1.5	9.3 ± 4.7
	103 ± 2	2.8 ± 1.9	15.0 ± 4.5	1.0 ± 0.3	4.1 ± 2.0	9.0 ± 4.0
	412 ± 16	3.6 ± 0.6	19.4 ± 6.6	1.7 ± 0.8	3.9 ± 3.0	25.5 ± 7.2
AC	55 ± 1	2.0 ± 1.0	10.0 ± 5.1	0.7 ± 0.4	3.0 ± 1.0	3.2 ± 0.6
	102 ± 7	1.9 ± 0.5	6.5 ± 1.7	0.2 ± 0.2	1.5 ± 0.6	6.7 ± 3.2
	341 ± 25	3.1 ± 1.9	17.3 ± 5.3	0.5 ± 0.3	2.1 ± 1.6	27.7 ± 14.1
III	56 ± 1	1.4 ± 1.2	17.9 ± 7.2	1.2 ± 1.1	2.3 ± 1.9	3.5 ± 2.6
	100 ± 1	1.9 ± 0.6	14.2 ± 1.9	2.4 ± 2.1	0.4 ± 0.2	7.0 ± 4.0
	318 ± 22	3.8 ± 3.7	24.3 ± 2.3	1.0 ± 0.8	1.2 ± 0.8	23.0 ± 3.3

[a] Corticosterone in μg/dl ± SE, $n = 5$.

ticosterone in favor of cortisol in this period of time with a stress of this magnitude and duration.

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