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β-Carboline-Induced Seizures in Mice: Genetic Analysis

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DESFORGES, C., P. VENAULT, R. H. DODD, G. CHAPOUTHIER AND P. L. ROUBERTOUX. *β-Carboline-induced seizures in mice: Genetic analysis.* PHARMACOL BIOCHEM BEHAV **34**(4) 733–737, 1989.—The inbred mouse strains BALB/cBy (C) and C57BL/6By (B6) differed significantly in their susceptibility to seizures induced by the benzodiazepine inverse agonist methyl β-carboline-3-carboxylate (β-CCM). Following a 5 mg/kg injection of β-CCM, 74% of C (n = 35) and 13% of B6 (n = 40) mice exhibited a convulsion. No sex difference was found. Analysis of the reciprocal F1s failed to show either maternal environmental and/or heterosomal effects. A genetic analysis of the strain difference in susceptibility to β-CCM-induced seizures using recombinant inbred strains (RIS) was performed. The strain distribution for the RIS showed a two group partition. Statistical analysis showed that, although a one-segregating-unit model could not be rejected to explain the strain difference in β-CCM-induced seizures, some of the evidence weakened the one-segregating-unit hypothesis.

Benzodiazepine Inverse agonist β-Carboline Methyl β-carboline-3-carboxylate (β-CCM) Seizures
Genetic analysis Inbred strains of mice (BALB/cBy and C57BL/6By) C × B recombinant inbred strains

BENZODIAZEPINES are a class of compounds with anticonvulsive, anxiolytic, sedative-hypnotic and muscle-relaxant properties (11), as well as amnesic properties (12). The detection in the central nervous system of specific, high-affinity receptors for these compounds which mediate their pharmacological effects (3, 4, 13) has allowed the discovery of other ligands which have intrinsic pharmacological properties that are opposite to those of benzodiazepines (5,16). These have been called "inverse agonists" (9). As far as convulsive properties are concerned, some of these inverse agonists are proconvulsant, while others are clearly convulsant. Both benzodiazepines and inverse agonists appear to exert their actions through the GABA receptor to which the benzodiazepine receptor is linked in a GABA-benzodiazepine-chloride channel receptor complex (10).

Differences in strain susceptibility to inverse agonists of the benzodiazepine receptor are known (15,23). Seale *et al.* (23) employed a classical Mendelian cross design (reciprocal F1 crosses, F2 crosses and backcrosses) using the inbred mouse strains SWR/J and CBA/J to investigate the genetic correlates of lethal susceptibility to seizures induced by the benzodiazepine inverse agonist methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM). They interpreted their results as indicating that these strain differences might be due to "a single autosomal gene determinant in which the allele specifying increased responsiveness is dominant to the allele determining hyporesponsiveness."

Our group has been involved in similar research with another inverse agonist, methyl β-carboline-3-carboxylate (β-CCM), and two different inbred strains of mice, BALB/cBy and C57BL/6By. In Swiss OF1 mice, β-CCM has been shown to be convulsant at high doses (5–10 mg/kg) (17), anxiogenic at moderate doses (1 mg/kg) (18), and to enhance performances in learning and memory tasks at low doses (0.3 mg/kg) (24).

METHOD

Subjects and Genetic Design

Experimental populations were composed of the two parental inbred strains of mice: BALB/cBy (C) and C57BL/6By (B6), their reciprocal F1 hybrids (CB6F1 and B6CF1; maternal parent strain mentioned first), and the 7 recombinant inbred strains (RIS) deriving from the progenitor strains (viz., CXBD, CXBE, CXBG, CXBH, CXBI, CXBJ, and CXBK) (2). The RIS will be referred to as D, E, G, H, I, J, and K, respectively.

Strains C and B6 were selected because they are genetically distinct: 41% of their tested genetic variants differ (19). Furthermore, a pilot experiment supplied evidence for differences in their reactivity to β-CCM. Their reciprocal F1s were tested to assess maternal environmental effects and the involvement of sex chromosomes. Possible differences between reciprocal F1 females would indicate maternal environmental effects. Should these be absent, the comparison of reciprocal F1 males would test for the presence of effects of the sex chromosomes (heterosomes). The 7 RIS were employed because they present various recombinations of alleles from their progenitor strains with specific recombinations fixed into each RIS. This method provides a logistically very efficient and feasible approach for testing a one-segregating-unit model underlying a particular trait. Such a model should not be

rejected if it is possible to dichotomize the strains from a RIS set as exhibiting the same phenotype as one or the other parental strain. The RIS distribution pattern (SDP) might then be compared with other identified SDPs (8). Pleiotropy or close genetic linkage might be expected if identical SDPs were found for two traits and, subsequently, the involved segregating-unit might be mapped (1,20).

The parental inbred strains and their RIS were maintained in our laboratory for two or three generations, using identified breeders from IFFA CREDO (Lyon, France) and CSEAL/CNRS (Centre de Sélection et d'Elevage des Animaux de Laboratoire/ Centre National de la Recherche Scientifique, Orléans, France). They derived, via Bailey, from the Jackson Laboratory colony from which they had been separated since 1980. At the beginning of the experiment, the parental strains and the RIS had been inbred for more than 160 and 94 generations, respectively.

Rearing Conditions

Animals were reared under standard conditions of 24 ± 0.5°C, a 12:12 hr photoperiod with lights on at 07:00 hr, water and Sourifarat (IMUAR) food available ad lib and dust-free sawdust bedding. From birth to weaning, they were maintained with only their mothers while sires were taken out of the mating cages one or two days before parturition. Offspring males were separated from sisters when weaned at 29 ± 2 days.

Drugs

β-CCM was synthesised by one of us (R.H.D.). It was dissolved in 0.2 N HCl (2 mg in 100 μl) and diluted to volume with saline. During all tests, 0.05 ml drug per 10 g body weight was administered IP. Each animal was employed only once. Dose-dependent effects were first studied in the parental strains, using 4 different doses in C mice (2.5, 3.75, 5 and 7.5 mg/kg) and 3 in B6 (all except 2.5 mg/kg). The dose of 5 mg/kg was finally selected for the genetic analysis. The final pH of these solutions was acidic and varied from 2 to 2.4.

Seizures

Animals were 11 ± 3 weeks old at the time of testing. Tests were performed in the rearing room from 11:00 to 15:00 hr. Three minutes before injection, individual mice were placed in $20 \times 42 \times 16$ cm large plastic cages littered with clean dust-free sawdust. Myoclonic jerks and vocalization were the initial symptoms of β-CCM action observed in some mice after its injection. "Reactive" mice had only one generalized seizure, succeeded by a variable period of behavioral suppression. Seizures and such behavior did not occur either after acid vehicle or after saline injections and no recurrent episode was noticed, confirming previous observations (17). Evolution of clinical symptoms as well as the number of animals convulsing were recorded. Seizures were considered as having occurred when full tonic-clonic convulsions were observed and the mouse fell on its flank. The observation period lasted up to 6 minutes from the time of injection. This length of time was chosen for the detection of seizure occurrence based on consideration of both the characterization of convulsions induced by β-CCM in Swiss OF1 mice (17) and pilot data concerning the two parental inbred strains C and B6.

Statistics

The Fisher exact probability test was used to compare the parental strains in the dose-dependent effect study, the sample size being too small to use the Chi-square test. The log likelihood ratio Chi-square test (G2) was used to compare the ratios of convulsing/ nonconvulsing mice in the genetic design, with $\alpha = 0.05$, where animals from the 5 mg/kg dose-response study were included.

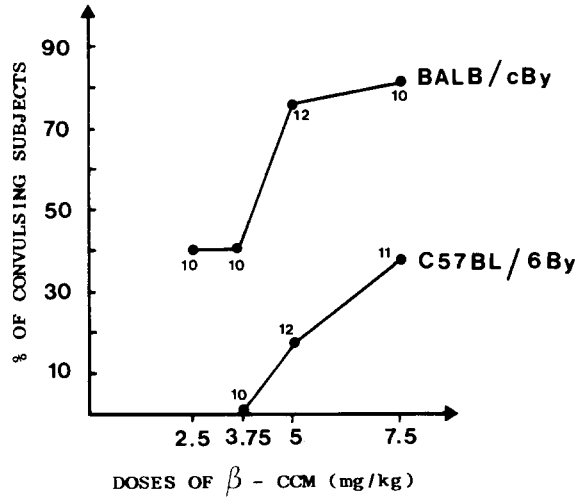

FIG. 1. Dose-dependent convulsant effects of β-CCM in BALB/cBy and C57BL/6By mice. For each point, the number of animals tested is indicated. Mortality: 5 mg/kg: 13% in C mice; 7.5 mg/kg: 30% in C and 9% in B6 mice.

RESULTS

Dose-Dependent Convulsive Effect of β-CCM in Strains C and B6

Figure 1 gives the percentages of convulsing animals in the parental inbred strains C and B6 for 3.75, 5 and 7.5 mg/kg of β-CCM injections, and, in C mice only, for 2.5 mg/kg. The 2.5 mg/kg dose was not injected in B6 mice because no convulsions occurred at 3.75 mg/kg. The occurrence of seizures in strains C and B6 increased with increasing doses of β-CCM.

The Fisher exact probability test was used to compare the parental strains at each of the tested doses. A dose of 5 mg/kg was finally chosen for subsequent experiments since it provided a significant difference between the two parental strains (C vs. B6: $p = 0.0058$), whereas no significant difference appeared for the dose of 7.5 mg/kg (C vs. B6: $p = 0.051$). This dose also seemed to be midway between maximum and minimum threshold doses, as no responses occurred in B6 mice at 3.75 mg/kg (C vs. B6: $p = 0.043$). Moreover, a dose of 7.5 mg/kg induced a drastic mortality in C mice (30%).

Strain Differences and Sex Effects

An overall likelihood ratio Chi-square G2 test including the parent strains and their reciprocal F1 hybrids showed a significant difference between these groups, $G2(3) = 25.97$, $p < 0.001$, which allowed the following single degree of freedom comparisons. The comparison between males and females of the two progenitor strains (Table 1) demonstrated no effect for sex [$G2(1) = 0.16$ for B6 and 0.93 for C, ns]. However, a strain effect was observed: significantly fewer B6 mice (13%) than C mice [74%; $G2(1) = 23.91$, $p < 0.001$] convulsed. To test for global maternal environmental effects, CB6F1 and B6CF1 females were compared. The difference between these two groups was not significant [CB6F1

TABLE 1

STRAIN AND SEX DIFFERENCES IN SUSCEPTIBILITY TO 5 mg/kg β-CCM-INDUCED SEIZURES IN BALB/cBy, C57BL/6By AND THEIR RECIPROCAL F1s, CB6F1 AND B6CF1 (MATERNAL PARENT STRAIN MENTIONED FIRST)

	BALB/cBy	C57BL/6By	CB6F1	B6CF1
♂	79%* (24)†	14% (29)	36% (22)	32% (25)
♀	64% (11)	9% (11)	40% (10)	31% (16)
Total	74% (35)	13% (40)	38% (32)	32% (41)

*% of convulsing mice.
†Number of tested mice.

vs. B6CF1 females: G2(1) = 0.21, ns]. When maternal environmental effects were not apparent, involvement of the heterosomes could be tested with CB6F1 and B6CF1 males. No such effects were evident, G2(1) = 0.10, ns. As there was no difference between males and females, data from both sexes were pooled for the subsequent comparisons. Reciprocal F1s did not differ [CB6F1 vs. B6CF1; G2(1) = 0.27, ns], and were also pooled. The percentage of seizing animals in the reciprocal F1 hybrids (n = 73) differed significantly from the percentage of both the two progenitor stocks (Table 2), but not from the mid-parental value, which is the mean of the two parents (43%) [mid-parental vs. F1 value: G2(1) = 0.73, ns]. The inheritance of reactivity to β-CCM in these strains appeared to be intermediate.

Genetic Analysis

An overall likelihood ratio Chi-square G2 test, including the parent strains and all 7 RIS, showed significant difference between strains, G2(8) = 44.49, $p<0.001$. Then the SDP was determined using 1 *df* G2 post hoc comparisons between the RIS and each of the parental strains (Table 2). This suggested that there were two sets of strains, each grouped with one of the parents and significantly different to the other: strains D, E, G, H, J, and K clustered with the more susceptible strain C (G2 values are presented in Table 2), whereas I was grouped with B6, G2(1) = 2.03, ns. The group of RIS which was not different from C, was then tested for homogeneity [C vs. D, E, G, H, J, K: G2(6) = 9.56, ns]. These results suggested a bipartition of the RIS, the two sets being grouped with one parent strain. The independence of these two groups was confirmed by comparing H (the less reactive in the set of reactive strains) with I, the RIS close to B6 [H vs. I: G2(1) = 3.86, $p<0.05$]. However, partial comparisons are needed in the reactive group since differences between the RIS are expected under a one-segregating-unit hypothesis (20). Differences were noticed between the higher responsive strains and the lower ones of the C group [D vs. H: G2(1) = 4.39, $p<0.05$; D vs. G: G2(1) = 3.86, $p<0.05$; K vs. H: G2(1) = 3.88, $p<0.05$].

DISCUSSION

Analysis of ratios of convulsing/nonconvulsing animals showed a significant difference between the highly inbred strains C and B6. The examination of the reciprocal F1s did not support the hypothesis of a maternal effect. However, the comparison of reciprocal F1s can only provide a rough information in respect to this effect since possible pre- and postnatal effects acting in opposite direction can cancel each other (21). It would be worthwhile to check this conclusion by the joint use of ovarian transplantation and fostering methods which provided accurate estimation of each effect (14). The difference between C and B6

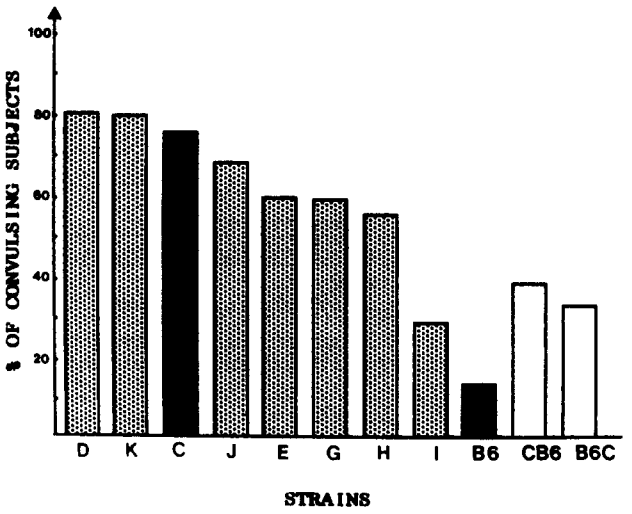

FIG. 2. Percentages of convulsions induced by a 5 mg/kg β-CCM IP injection, in BALB/cBy and C57BL/6By mice, their reciprocal F1s and their 7 recombinant inbred strains. Dark columns: parental strains; white columns: reciprocal F1s; and grey columns: RIS.

could be correlated to genetic factors since the classical F1 comparisons did not permit a maternal effect to be considered. Neither sex association (females and males did not differ), nor sex-linked inheritance (no difference between reciprocal F1s males) was present, suggesting an autosomal inheritance of susceptibility to β-CCM-induced seizures, measured by occurrence of convulsions. The pooled value of the F1s compared to the parent strains did not provide evidence for an heterotic effect often associated with polygenic inheritance (6). It indicated here that the trait is intermediately inherited (no difference with the mid-parent value). The genetic analysis using the RIS could suggest a

TABLE 2

CONVULSING/NONCONVULSING RATIOS, FOLLOWING A 5 mg/kg β-CCM IP INJECTION IN THE PARENTAL STRAINS BALB/cBy AND C57BL/6By, THE 2 RECIPROCAL F1s AND THE 7 RECOMBINANT INBRED STRAINS

Tested Population	Convulsing/Non-convulsing Ratios	G2 (1 *df*) vs. BALB/cBy	G2 (1 *df*) vs. C57BL/6By
Parental			
BALB/cBy	26/9 (74%)	—	23.91†
C57BL/6By	5/35 (13%)	23.91†	—
RIS			
CXBD	26/7 (79%)	0.19	25.90†
CXBE	14/10 (58%)	1.63	13.02†
CXBG	30/22 (58%)	2.47	16.56†
CXBH	19/16 (54%)	2.99	13.05†
CXBI	6/16 (27%)	11.01†	1.23
CXBJ	18/9 (67%)	0.43	17.62†
CXBK	22/6 (79%)	0.16	23.90†
Reciprocal F1s			
CB6 F1	12/20 (38%)	13.95†	5.78*
B6C F1	13/28 (32%)		

The two last columns give the G2 values for comparisons between the parental strains and other genotypes.
*$p<0.05$, †$p<0.001$.

one-segregating-unit correlate for reactivity to β-CCM in the B6, C and CXB populations; a high reactive and a low reactive set of strains were detected, each grouped with one of the parent. However, differences have been pointed out between the extremes of the reactive group and, thus, consequently weakened the one-segregating-unit correlate conclusion. This particular RIS pattern would suggest a more complex model involving one segregating-unit with a major effect plus segregation-units with minor effects. Present data did not permit a conclusion and further experiments, employing different genetic techniques (classical Mendelian crosses), are needed.

Previously published results (15,22) have provided evidence for genetic correlates of behavioral responses to benzodiazepines. A successful response to bidirectional artificial selection to diazepam reactivity was carried out by Gallaher *et al.* (7). Presence of response after the first generations was compatible with several segregating-unit correlates, whereas Seale *et al.*'s (23) conclusions favor a one-segregating-unit hypothesis.

This apparent discrepancy is not surprising. The genetic correlates of a trait could depend on the genetic structure of the population. Moreover, differences in procedures such as the nature of β-carbolines (DMCM vs. β-CCM), the drug vehicle (Tween vs. HCl), measures of the reactivity (mortality vs. convulsion), and particularly the strains in which the analysis has been performed could explain this discrepancy. Genetic analysis can identify genes involved in the reactivity to β-carbolines and/or genes involved in the trait selected for the measurement of reactivity. Finally, the results of Seale *et al.* and our own are not incompatible with identical sensitivity in CBA/H and SWR/J strains, the death in these strains being the result of an increased liability to breathing and cardiac disorders that would be expressed as a consequence of modifications induced by DMCM.

The question thus remains as to whether different segregating-units are involved in these two different studies. Three different genetical mechanisms might explain this discrepancy. First, different segregating units might be involved in these two studies. Second, more than two different allelic forms of the same gene with major effect might exist at a single locus (polymorphism). Finally, interactions with the genetic background might cause these different levels of dominance. Further experiments are needed to clarify this point.

The question of whether the differences found between strains of mice are related to the specific effects of β-carbolines on their receptors or whether indirect mechanisms or pharmacokinetic variables such as metabolism or biodistribution are involved, also merit closer investigation. The only extensive investigation of pharmacokinetic variables involved in different sensitivity to seizures of two stocks of mice (NIH general purpose and NIH) has been reported by Schweri *et al.* (22). These authors attributed the distinct levels in susceptibility to differences in brain levels of β-CCM. In contrast, Nutt and Lister (15) suggested, based on indirect evidence, that genetic determinants directly involve benzodiazepine receptor functions. Similarly, Seale *et al.* (23) reported that preliminary experiments seemed to rule out an explanation of their results by different metabolism or biodistribution of DMCM. This important question remains unanswered for the moment and will be the subject of further study.

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