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# Coincidence of Seizure Susceptibility to Caffeine and to the Benzodiazepine Inverse Agonist, DMCM, in SWR and CBA Inbred Mice

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SEALE, T. W., J. M. CARNEY, O. M. RENNERT, M. FLUX AND P. SKOLNICK. *Coincidence of seizure susceptibility to caffeine and to the benzodiazepine inverse agonist, DMCM, in SWR and CBA inbred mice.* PHARMACOL BIOCHEM BEHAV 26(2) 381–387, 1987.—Several lines of evidence suggest that the convulsant actions of caffeine are mediated through benzodiazepine receptors. A pharmacogenetic approach has been used to further explore the relationship of these receptors to caffeine-induced seizures. The susceptibility of two inbred strains of mice (CBA and SWR) to the convulsant actions of picrotoxinin, strychnine, Ro 5-4864 and DMCM was examined. Previous studies have demonstrated these two strains differ in their susceptibilities to the convulsant action of caffeine. While no differences were observed between these two strains in susceptibility to tonic seizures induced by picrotoxinin, R0 5-4864 or strychnine, SWR mice were significantly less sensitive to tonic seizures induced by DMCM compared to CBA mice ($CD_{50}$ values in CBA and SWR mice were 6 and 12 mg/kg IP). Both clonazepam and the benzodiazepine receptor antagonist, Ro 15-1788, significantly blocked caffeine-induced seizures. Further, when subconvulsant doses of caffeine and DMCM were combined, a synergistic action was observed. Taken together, these findings support the hypothesis that the convulsant actions of caffeine result from an action at benzodiazepine receptors, and that the hyporesponsiveness of the SWR strain to both caffeine- and DMCM-induced seizures could result from an inherited abnormality in these sites.

Methylxanthines Caffeine Theophylline β-Carbolines Benzodiazepine inverse agonist
Picrotoxinin Strychnine Seizures Inbred mice Behavioral genetics

MANY drugs possessing anticonvulsant (e.g., benzodiazepines, cyclopyrrolones, barbiturates) and convulsant (picrotoxinin, pentylenetetrazole) actions exert these effects through the benzodiazepine-γ-aminobutyric (GABA) receptor-chloride ionophore complex [2, 9, 14, 20, 24, 34]. High doses of methylxanthines such as caffeine and theophylline produce convulsions, which is a particular concern in the therapeutic use of theophylline as a bronchodilator [22, 31, 41]. Although the mechanism by which methylxanthines elicit convulsions is unclear [7, 13, 28], several lines of evidence suggest this effect may be produced by occupation of "central type" benzodiazepine receptors. Both caffeine and theophylline competitively inhibit [$^3$H] diazepam binding to benzodiazepine receptors *in vitro*, and the $IC_{50}$ of caffeine is well within the concentration range which can be achieved *in vivo* [18]. Caffeine-induced seizures can also be antagonized by another purine, inosine, which has been proposed to be an endogenous benzodiazepine receptor ligand [17,33]. Furthermore, Veluci and Webster [37] have reported that the benzodiazepine receptor antagonist, Ro 15-1788, can block caffeine-induced seizures, which suggests that caffeine may act in a manner similar to a benzodiazepine receptor "inverse agonist." Purines interact with "central type" but "peripheral type" benzodiazepine binding sites [32]. Nonetheless, methylxanthines such as caffeine affect diverse neurochemical pathways at concentrations substantially lower than required to influence benzodiazepine receptors (e.g., antagonism of adenosine receptors, inhibition of phosphodiesterases, alteration of calcium mediated events, release of brain catecholamines [1, 6, 7, 11, 12, 16]).

A unique analytical approach to determine whether caffeine induces seizures through its action on benzodiazepine receptors involves the characterization of mutants with al-

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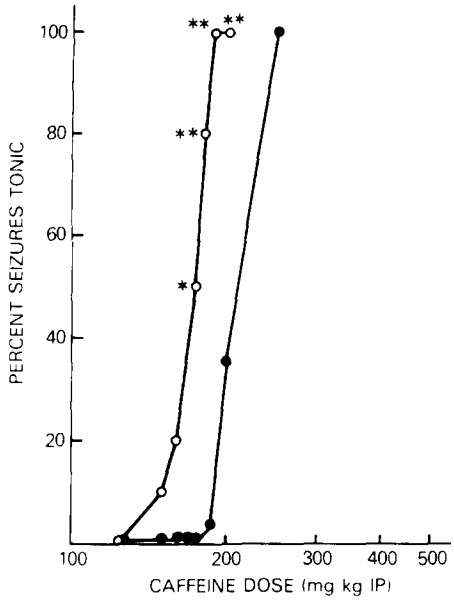


FIG. 1. Dose dependency of stress-potentiated, caffeine-induced tonic seizures and death in CBA and SWR inbred mice. Ten animals were used for each dose and compared in a pairwise fashion. (○) CBA mice; (●) SWR mice; responses which differ significantly from one another are indicated by asterisks (*$p<0.05$; **$p<0.001$).

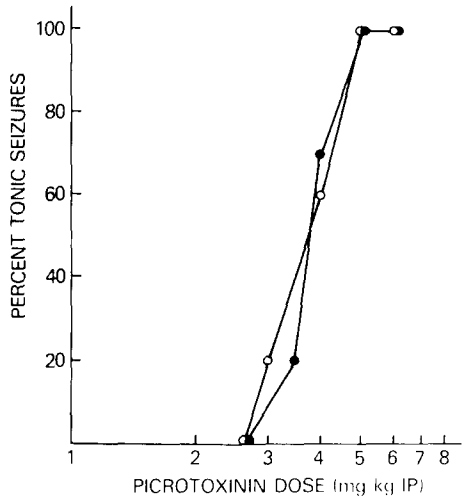


FIG. 2. Dose dependence of picrotoxinin-induced tonic seizures and death in CBA and SWR inbred mice. At each dose the response of 10 mice from each strain was compared in a pairwise fashion. No difference in the responsiveness of the two inbred strains was noted. (○) CBA mice; (●) SWR mice.

tered susceptibility to the convulsant action of caffeine. If the convulsant action of caffeine does involve the benzodiazepine receptor, mutants with altered susceptibility to caffeine also would be expected to have altered behavioral responses to central type benzodiazepine receptor ligands. Recently we have reported that different strains of inbred mice exhibit markedly different behavioral responses to acute administration of toxic levels of caffeine [28]. This altered behavioral responsiveness to caffeine also extends to other structurally related methylxanthines [15]. SWR and CBA are two strains found to be different in their inherent responsiveness to high doses of caffeine. For example, 187 mg/kg IP of caffeine caused tonic seizures and death in all CBA mice, without producing convulsions or death in SWR mice. Blood levels of caffeine were indistinguishable between the two strains, a finding which suggested that pharmacodynamic differences were unlikely to account for these differences in susceptibility to caffeine-induced seizure susceptibility [5]. This strain-specific difference in susceptibility to caffeine appears to be inherited as a single gene trait in which caffeine responsiveness is dominant to hyporesponsiveness [29]. Given the possible involvement of central type benzodiazepine receptors in the convulsant action of caffeine, we investigated the behavioral responsiveness of these two strains to several chemical convulsants known to act by different neurochemical mechanisms. We now report that the difference in convulsant sensitivity between these two inbred stains of mice is pharmacologically specific such that altered susceptibility to caffeine-induced seizures is coincidently associated with altered responsiveness to a central type benzodiazepine inverse agonist, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) [4, 21, 26], but not other convulsants including picrotoxinin, strychnine or Ro-5-4864. These findings suggest a specific genetic locus which may determine sensitivity to benzodiazepine receptor ligands.

## METHOD

### *Animals*

Adult male mice of inbred strains CBA/J and SWR/J (Jackson Laboratory, Bar Harbor, ME) nine weeks of age were housed in groups of 6 animals per cage on a continuous 12 hour light-dark cycle under constant humidity and temperature (19–21°C). Animals were drug naive and were used for only a single administration of drug. The litter used was hardwood chips (Beta-Chip, Northeastern Products Corp., Wanesburg, NY). Free access to a standard rodent pellet food (NIH-07, AGWAY Inc., St. Marys, OH) and water was given.

### *Source of Drugs and Preparation of Solutions*

Caffeine, picrotoxinin and strychnine were obtained from Sigma Chemical Co. (St. Louis, MO). Ro 5-4864, Ro 15-1788 and clonazepam were generously provided by Hoffmann-LaRoche (Nutley, NJ). PK 11195 was generously provided by G. LeFur, PHARMUKA Laboratories (Gennervilliers, France). DMCM was supplied by Research Biochemicals (Wayland, MA). Caffeine solutions were freshly prepared in physiological saline containing 5 mM NaOH. Picrotoxinin was dissolved in hot physiological saline; the freshly prepared solution was cooled to room temperature and was injected immediately. Strychnine, Ro 5-4864, PK 11195, Ro 15-1788 and clonazepam were dissolved in a 1:1 mixture by weight of dimethylsulfoxide (Fisher Scientific, Fairlawn, NJ) and Emulphor (Emulphor EL-620, GAF Corp, New York, NY) and then diluted with physiological saline to give a final vehicle composition of 30% dimethylsulfoxide-Emulphor to 70% saline. These solutions were prepared immediately before injection.

### *Seizure Susceptibility Testing*

The dose dependent action of caffeine was assessed with the same quantitative method for stress potentiation of caffeine-induced tonic seizures which we used originally to

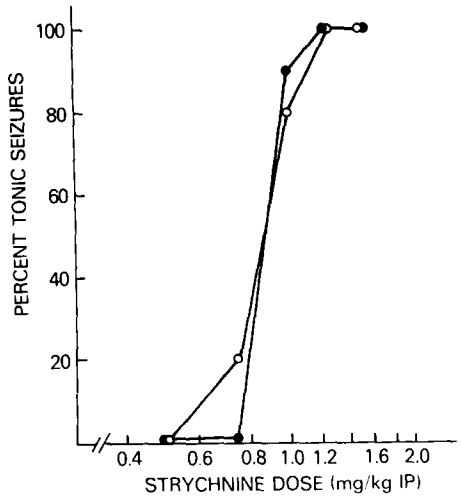


FIG. 3. Dose dependence of strychnine-induced tonic seizures and death in CBA and SWR inbred mice. At each dose the response of 10 mice from each strain was compared in a pairwise fashion. No significant difference in the responsiveness of the two inbred strains to this convulsant was noted. (○) CBA mice; (●) SWR mice.

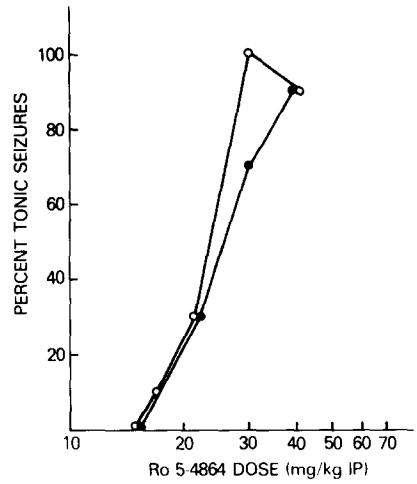


FIG. 4. Dose dependence of Ro 5-4864-induced tonic seizures and death in CBA and SWR inbred mice. At each dose the response of 10 mice from each strain was compared in a pairwise fashion. No significant difference in responsiveness of the two inbred strains to this convulsant was noted. (○) CBA mice; (●) SWR mice.

identify the differences in caffeine susceptibility between SWR and CBA mice [28,29]. A swim stress, achieved by gently placing individual animals in a large beaker of water, gave reproducible results which could be quantitated in terms of time of onset and frequency of tonic seizures and death. Twenty minutes after caffeine administration, mice were subjected to a swimming stress by placing individual animals in a 2 liter beaker containing water at 25°C. Untreated or nonresponding animals swam actively for >2 minutes and did not have tonic seizures or die. Inactive animals floated. A test on an individual animal was scored as positive when seizures and death occurred in <2 minutes. At least 10 animals were scored for responsiveness at each caffeine dose.

Tonic seizures and death scored in individual animals (n=10 for each dosage) according to the behavioral description of Seyfried [30], was determined for 30 minutes following intraperitoneal administration of each of the other convulsants—picrotoxin, strychnine, Ro 5-4864 and DMCM. In recognition of the well known diurnal and temperature effects on convulsant sensitivity, experiments were conducted from 0900 to 1700 hours at 19–21°C. Dosing and strains were staggered so as to avoid inadvertant selection for strain differences in diurnal rhythm. The occurrence of a tonic seizure was scored as positive when all four legs of an animal were rigidly extended to the rear. Respiratory arrest usually but not always followed the occurrence of a tonic seizure. All comparisons of dose dependent responses between the two strains were made directly by simultaneously injecting mice from both strains with the same drug solutions.

### *Statistical Testing*

Comparision of the responses of these mouse strains to individual convulsants at specific doses was performed by the Fisher exact method [10]. A value of $p<0.05$ was taken as statistically significant.

## RESULTS

### *Comparison of the Dose Dependence of Caffeine-Induced Tonic Seizures Between CBA and SWR*

The dose dependent difference between SWR and CBA mice in susceptibility to caffeine-induced, stress-potentiated seizures is shown in Fig. 1. At doses of 160–200 mg/kg IP, a significantly greater fraction of CBA mice have tonic convulsions and die. While the greater susceptibility of CBA mice to tonic convulsions and death is first distinguished at 160 mg/kg IP, it becomes significant at 170 mg/kg IP (50% seizures and death in CBA *versus* no effect on SWR, $p<0.05$) and even more striking at doses between 175 and 187 mg/kg IP (e.g., 100% seizures and death in CBA at 187 mg/kg IP *versus* <5% in SWR mice, $p<0.001$). Under the conditions used here, mice of both strains seize and die at caffeine doses of ≥200 mg/kg IP. These new data are in accord with our previous incomplete dose dependency data by which we first identified and subsequently characterized the inheritance of this interstrain difference [28].

### *Specificity of the Convulsant Susceptibility Difference Between CBA and SWR Mice*

To determine if this difference in susceptibility to caffeine-induced seizures and death was specific, we investigated the effects of chemical convulsants with different neurochemical sites of action in these two strains. Agents tested included picrotoxinin, strychnine, Ro 5-4864, and DMCM. The comparative dose response curves in the SWR and CBA strains for the induction of tonic seizures by these convulsants are shown in Figs. 2–4. Figure 2 shows that both inbred strains of mice were equally responsive to picrotoxinin over a range of doses from 2.5 to 7 mg/kg IP. Although they were significantly more susceptible to the convulsant action of strychnine (Fig. 3), SWR and CBA mice were also equally responsive to tonic seizures induced by this agent over the range of doses from 0.5 to 1.6 mg/kg IP. Similarly, adminis-

TABLE 1

SUMMARY OF RELATIVE SUSCEPTIBILITIES OF CBA AND SWR INBRED MICE TO VARIOUS CHEMICAL CONVULSANTS

| Convulsant | $CD_{50}$ CBA | $CD_{50}$ SWR | Difference in Relative Susceptibility |
|---|---|---|---|
| caffeine | 170 | 210 | CBA>SWR |
| strychnine | 0.88 | 0.88 | none |
| picrotoxinin | 3.8 | 3.8 | none |
| Ro 5-4864 | 24 | 26 | none |
| DMCM | 6 | 12 | CBA>SWR |

$CD_{50}$ values are expressed in mg/kg IP. These values are estimated from Figs. 1–5.

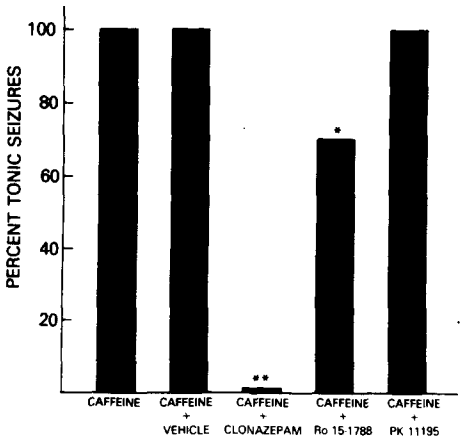


FIG. 6. Stress potentiated tonic seizures and death induced by caffeine in CBA mice in the presence and absence of a benzodiazepine agonist and antagonists. For the treatment with caffeine alone, 20 mice were tested. Ten animals were used for vehicle or benzodiazepine pre-treatments. Clonazepam was administered at a dose of 1 mg/kg IP, Ro 15–1788 at a dose of 10 mg/kg IP and PK 11195 at a dose of 20 mg/kg IP. Statistically significant differences caused by pre-treatment are marked with an asterisk (*$p<0.05$; **$p<0.0001$).

tration of the convulsant Ro 5-4864 produced tonic convulsions in a dose dependent manner over the range of 20–50 mg/kg IP, but both strains responded in a manner indistinguishable from one another (Fig. 4). The potencies of these convulsants estimated from Figs. 1–4 are compared to caffeine in the two mouse strains and summarized in Table 1.

In contrast to the lack of differential convulsant action of picrotoxinin, strychnine and Ro 5-4864 on the SWR and CBA strains, the dose dependent ability of DMCM to induce convulsions in the two strains differed significantly. As with caffeine, CBA mice were more susceptible than SWR mice to tonic seizures induced by DMCM (Fig. 5). The difference in responsiveness to DMCM between the strains resulted in a two-fold change in estimated $CD_{50}$ (Fig. 5, Table 1).

*Interaction Between Caffeine and Benzodiazepines Receptor Ligands*

To further characterize the putative interaction of caffeine with "central type" benzodiazepine receptors, the interactions between caffeine and the "central type" benzodiazepine agonist, clonazepam, the benzodiazepine receptor antagonist, Ro 15-1788, and DMCM were investigated in CBA mice. Figure 6 shows that pre-treatment with both clonazepam (1 mg/kg IP) and Ro 15-1788 (10 mg/kg IP) prior to the administration of a $CD_{100}$ dose of caffeine (187 mg/kg IP) significantly reduced the number of animals having convulsions (respectively $p<0.0001$ and $p<0.05$). Clonazepam completely blocked caffeine-induced seizures. Ro 15-1788 only partially blocked the convulsant action of caffeine.

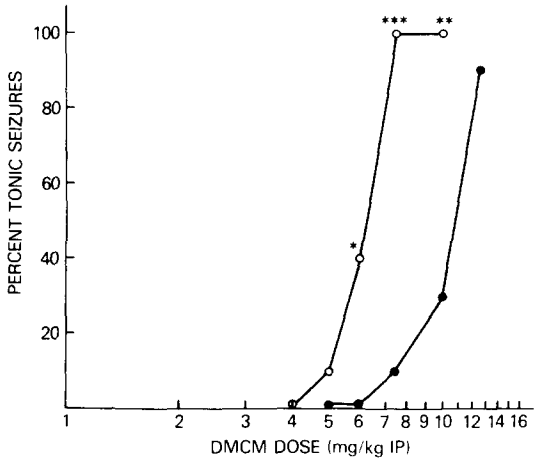


FIG. 5. Dose dependence of the central benzodiazepine inverse agonist, DMCM-induced tonic seizures and death in CBA and SWR inbred mice. At each dose the response of 10 mice from each strain was compared in a pairwise fashion. Statistically significant differences in susceptibility to a given dose between the strains are marked with an asterisk (*$p<0.05$; **$p<0.001$; ***$p<0.0001$). (○) CBA mice; (●) SWR mice.

Another approach to the interaction of caffeine with central type benzodiazepine receptors involves the behavioral interaction between subconvulsant doses of a benzodiazepine inverse agonist and a subconvulsant dose of caffeine. The induction of seizures by combined subconvulsant doses of DMCM and caffeine is shown in Fig. 7. A subconvulsant dose (150 mg/kg IP) of caffeine was injected into CBA mice together with a subconvulsant dose (4 mg/kg IP) of DMCM. While neither drug alone produced convulsions at this dose, a statistically significant ($p<0.05$) increase in the percentage of mice convulsing was found when the two convulsants were combined.

## DISCUSSION

Several lines of evidence suggest that the convulsant actions of caffeine are mediated via central benzodiazepine receptors. Marangos *et al.* [17,18] have shown that methylxanthines such as caffeine competitively inhibit [$^3$H] diazepam binding to benzodiazepine receptors in vitro. Furthermore, these investigators demonstrated a dose dependent blockade of caffeine-induced seizures by inosine, which has been proposed to be endogenous ligand of the benzodiazepine receptor [33]. Velucci and Webster [37] have shown that the benzodiazepine receptor antagonist Ro 15-1788 reduces the convulsant actions of caffeine which suggests that caffeine could function as a benzodiazepine receptor inverse agonist. Caffeine has a low affinity for ben-

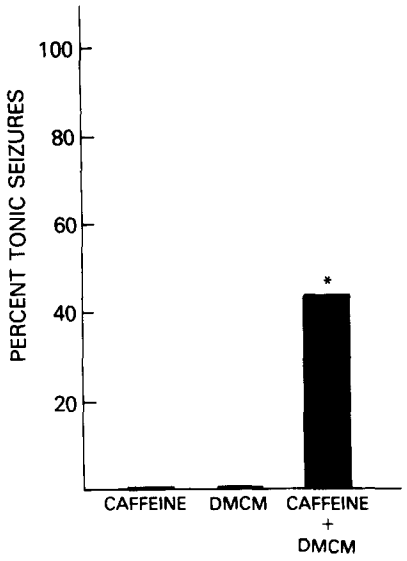


FIG. 7. Tonic seizures and death induced by the interaction of subconvulsant doses of caffeine and DMCM in CBA mice. Fifteen mice were treated with a subconvulsant dose of caffeine alone (150 mg/kg IP) and 10 each were treated with DMCM (4 mg/kg IP) or the combination of the two drugs. The combined administration of the two drugs caused a significant increase in the frequency of convulsions ($*p<0.05$) compared to the drugs administered singly.

zodiazepine receptors (284 $\mu$M) [18], although this concentration of caffeine could be achieved after *in vivo* administration. Nonetheless, caffeine has a number of documented actions on other neurotransmitter systems at concentrations well below those that affect the benzodiazepine receptor. Although blockade of a pharmacologic action by Ro 15-1788 is generally accepted as indicating a benzodiazepine-receptor mechanism of action, the dose of Ro 15-1788 capable of partially antagonizing caffeine-induced convulsions [37] has been reported to produce a partial agonist action in some pharmacologic paradigms (e.g., [8]).

Thus, we attempted to clarify the mechanisms by which caffeine produces convulsions using a pharmacogenetic approach with two strains of inbred mice previously shown [28,29] to have the differences in susceptibility to the convulsant actions of caffeine. The agents used, DMCM, picrotoxinin, Ro 5-4864, and strychnine, all have defined neurochemical sites of action. Both picrotoxinin and Ro 5-4864 have been shown to bind to sites at or near GABA-gated chloride channels [35, 36, 38, 39]. Strychnine has well documented actions as a glycine antagonist [34] while DMCM binds to benzodiazepine receptors to produce its convulsant effects which can be blocked by Ro 15-1788 [3,4]. The observation of a coincidence in seizure susceptibility to caffeine and DMCM, but not the other convulsants (Table 1, Figs. 1–5) suggests that a single mutational change may underlie this apparently specific alteration in seizure susceptibility since coincident changes in convulsant sensitivity would not be expected unless these agents acted by a common mechanism. This mutation is envisioned to reside in a gene specifying a component of the GABA receptor-benzodiazepine receptor-chloride ionophore complex, probably in a structural gene encoding the production (number) and/or function of a subset of benzodiazepine receptors. An allelic difference at a single gene locus has been shown to account for the change in susceptibility to caffeine-induced seizures between SWR and CBA mice [29]. However, the identity of this gene with that controlling relative susceptibility to DMCM has yet to be established in these strains. Because no significant differences in methylxanthine metabolism and compartmentation have been found between these strains [15] and because DMCM is not readily catabolized compared to other $\beta$-carbolines [27], it is probable that pharmacodynamic differences between the SWR and CBA strains do not account for these changes in convulsant sensitivity.

The observation that these strains are equally sensitive to convulsants thought to act near or at the $GABA_A$ receptor-gated chloride ionophore, picrotoxinin and Ro 5-4864 [24, 25, 35, 36, 39], suggests that an alteration in this chloride ionophore would not account for the differences in convulsant sensitivity to DMCM and caffeine, despite the role of the chloride ionophore as the "effector" component of the benzodiazepine receptor. Further, lack of a difference in sensitivity to picrotoxinin (and Ro 5-4864) but the occurrence of a difference in sensitivity to DMCM and caffeine strongly suggests that the components of the supramolecular complex may be genetically regulated in an independent manner. Several possible mechanisms to explain the observed differences in convulsant sensitivity between the strains can be excluded by comparing available data with theoretical expectations. A genetically determined change in the level of endogenously occurring benzodiazepine ligand(s) such as inosine [17,18] is expected to cause a non-specific alteration in susceptibility to seizures induced by a variety of pharmacological agents through a generalized mechanism involving activation of GABA-mediated inhibition. This type of neurochemical change is ruled out because of the pharmacological specificity of the change in seizure susceptibility that we observed. It also is inconsistent with the dominance of caffeine responsiveness, rather than hyporesponsiveness, in $F_1$ hybrid animals [29]. GABA content of brain nerve endings is known to alter pharmacologically-induced seizure thresholds [16]. A difference in GABA level, GABA receptor number or the affinity of this receptor for GABA can be ruled out for the same reasons stated above. Further, the occurrence of neurochemical alterations of this type are expected to be associated with changes in sensitivity to bicuculline, a direct-acting GABA receptor antagonist [2,20]. No difference in the dose dependence of bicuculline-induced tonic seizures has been observed between these mouse strains (Seale, Roderick and Skolnick, manuscript in preparation). Finally, if endogenous GABA levels or GABA receptor binding differed significantly between these two strains of mice, a marked difference in picrotoxinin induced tonic seizures between them is expected to occur because the picrotoxinin binding site is a component of the GABA-gated chloride channel [20, 25, 35]. No such difference in picrotoxinin responsiveness was observed. Similarly a change in the structure or coupling of the chloride ionophore/picrotoxinin binding site to the GABA receptor might be expected to result in a generalized change in convulsant sensitivity and ought to alter responsiveness to seizures induced by picrotoxinin and/or bicuculline [20, 25, 35, 40]. Because no differences in susceptibility were observed, it is improbable that such a neurochemical alteration has occurred in these strains of mice. Having eliminated these three possibilities, we are left with the possibility of a neurochemical alteration in the benzodiazepine receptor.

An inherent reduction in benzodiazepine receptor

number, a decrease in affinity for benzodiazepine ligands or an uncoupling of the benzodiazepine binding sites from GABA receptors could account for the observed coincident hyporesponsiveness of SWR mice to caffeine and DMCM. Such changes are not expected to have a generalized effect on convulsant sensitivity if endogenous benzodiazepine "tone" is low in the normal (non-mutant, non-stressed) state. Our data showing pharmacological specificity of the change in convulsant sensitivity are in keeping with this expectation. Also consistent with this hypothesis is the dominance of convulsant susceptibility, rather than hyporesponsiveness, in $F_1$ hybrid derivatives of these two strains [29]. Additional support for this hypothesis comes from our observation that the hyporesponsiveness of SWR mice to DMCM and caffeine appears not to be restricted to benzodiazepine inverse agonists. This strain also is hyporesponsive to diazepam-induced impairment of rotorod performance compared to the CBA strain (Seale and Skolnick, manuscript in preparation). Preliminary membrane binding studies employing [$^3$H] flunitrazepam have identified no differences in specific benzodiazepine binding in forebrain, striatum or cerebellum between these two strains (Seale and Skolnick). Others have shown that the coupling of components of the GABA receptor-benzodiazepine receptor-chloride ionophore complex can be subtly altered both *in vivo* and *in vitro* manipulations [19,23]. For example, repeated DMCM treatment leads to enhanced ability of GABA to reduce [$^3$H] DMCM binding to cortical neuronal membranes without affecting baseline [$^3$H] diazepam binding or [$^{35}$S] TBPS binding to chloride channels [19]. Taken together these data suggest that the differences in convulsant responsiveness between the two strains of mice may arise from an inherent change in coupling of the benzodiazepine receptor to the GABA receptor.

An alternative explanation for the occurrence of hyporesponsiveness to both caffeine and DMCM in SWR mice is that two separate mutations are present in this strain, one of which specifically alters caffeine susceptibility and the other of which specifically affects susceptibility to the benzodiazepine inverse agonist. Thus, hyporesponsiveness to these two convulsants would be coincidental to two genotypic changes, rather than a coincident phenotypic effect arising from a single genetic change between these two strains of mice. These two hypotheses can be experimentally distinguished by genetic studies of crosses between SWR and CBA strains in which the co-segregation of these two susceptibilities is examined in backcross progeny.

In summary, the data presented in this study lend further pharmacological and genetic support to the likelihood that central benzodiazepine receptors are involved in the mediation of methlxanthine-induced seizures. Even if subsequent genetic experiments establish that separate genes encode convulsant responsiveness to caffeine and DMCM, the present data established that strain specific differences in responsiveness to central type benzodiazepine inverse agonists do occur in inbred strains of mice. Such mutations may have significant value for evaluating intrinsic and environmentally-induced behavioral effects which are subject to modification by central type benzodiazepines.

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