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Influences of Genotype and Sex on Behavioral Tolerance to Nicotine in Mice[1]

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HATCHELL, P. C. AND A. C. COLLINS *Influences of genotype and sex on behavioral tolerance to nicotine in mice.* PHARMAC. BIOCHEM. BEHAV. 6(1) 25–30, 1977. – Spontaneous motor activity in a Y-maze was measured in DBA/2Ibg and C57BL/6Ibg mice which had received nicotine or saline injections three times a day for two, four or seven days. Both genotype and sex influenced the development of tolerance to nicotine's effects on spontaneous motor activity, with DBA males requiring the longest exposure to nicotine and C57 males requiring the shortest drug exposure for tolerance development. DBA and C57 females developed behavioral tolerance equally after two days of pretreatment, but the C57 females showed a greater degree of tolerance after seven days of injections than did the DBA females. The development of behavioral tolerance in DBA males after four days of nicotine pretreatment was associated with the development of drug dispositional tolerance, with minimal evidence for a change in nervous system sensitivity. Drug dispositional tolerance in DBA females, C57 males and C57 females, however, did not seem to affect spontaneous motor activity.

Nicotine Genotype influence Sex influence Mice Behavioral tolerance Functional tolerance Drug dispositional tolerance

TOLERANCE is the state in which a gradual decrease in the effect of a drug is produced by its repeated administration, or in which a gradual increase in the dosage of the drug is necessary to cause the same effect as that produced by the initial dose. Tolerance to nicotine's effects has been demonstrated in investigations of learned behavior [4, 17, 20, 22], EEG and behavioral arousal [3, 5, 9, 25, 27] and motor activity [12, 19, 23, 24].

Little attention has been given to genotype or sex in studies of nicotine tolerance; many authors do not indicate the strain or even the sex of the animals in their experiments. It is becoming increasingly apparent, however, that these parameters cannot be ignored in behavioral, biochemical or pharmacological studies. Differences among inbred strains of mice, for instance, are well known for alcohol-related behavior [15,16] and alcohol biochemistry [11,14], and influences of genetic factors or sex have been shown for behavioral response to nicotine [1, 2, 7, 8, 18], nicotine metabolism in liver [21] and neuronal sensitivity to nicotine (research in preparation from this laboratory).

The present study was designed to investigate the influences of genotype and sex on the development of behavioral tolerance to nicotine in mice. The development of tolerance to nicotine's effects on spontaneous motor activity was assessed in a Y-maze, and nicotine concentration in liver and brain were measured to determine if changes in the rate of liver metabolism or brain nicotine content were associated with tolerance development. The use of two inbred mouse strains allowed the assessment of both genetic and sex influences on these measures.

METHOD

Animals

Male and female mice of the C57BL/6Ibg and DBA/2Ibg inbred strains were maintained at the Institute for Behavioral Genetics, University of Colorado, in a colony room with a standard temperature of 72 ± 3°F and a 24-hr light–dark cycle (equal periods of light and dark). Three same-sex animals were housed in each cage, and access to food and water was unrestricted. All animals were 55 ± 5 days of age at the beginning of the experiment.

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Drug Treatment

For behavioral testing, nicotine base was dissolved in distilled water to a concentration of 0.1 mg base/ml, and 1 mg/kg body weight was injected intraperitoneally. Control animals were injected intraperitoneally with physiological saline (0.9% NaCl) at a dose of 10 ml/kg body weight. For biochemical testing, ^{3}H-nicotine bitartrate (Amersham/Searle) was dissolved in distilled water to a concentration of 0.1 μCi/0.1 ml solution. Unlabeled nicotine was added to achieve a final concentration of 0.1 mg/ml and was administered as described above. This nicotine concentration was chosen for behavioral and biochemical assessments after testing the effect of several different nicotine concentrations on Y-maze spontaneous motor activity. The lowest nicotine concentration which produced consistent depressant effects on motor activity was 1 mg/kg.

Motor Activity Measurement

A symmetrical, Y-shaped runway, 10.2 cm high with arms 26.0 cm long and 6.1 cm wide, was constructed from opaque, black Plexiglas. Hinged covers over each arm and over the center section where the arms join were made of clear, red Plexiglas. Each of the three arms of the runway was divided by lines marked on the cover into two 13.0 cm sections. A 75 W light bulb, 111.8 cm above the center of the maze, provided illumination for observation of animals within the apparatus.

Each animal was tested 5 min after receiving an injection of either nicotine or saline. The animal was placed in the center of the runway, all covers were closed, and movement of the animal from one section to another (all four feet crossing a dividing line) was recorded for 3 min. Activity was expressed as the number of maze sections entered during the 3-min period. All mice were experimentally naive when tested.

Estimation of Nicotine

Animals were decapitated 5 min after receiving an injection of radio-labeled nicotine. The liver and brain were immediately removed, rinsed with cold saline, blotted and weighed. Tissues were homogenized in 5 volumes of cold saline and centrifuged at 9500 rpm for 10 min. A 1.5 ml aliquot was pipetted into 1 ml of 0.1 N NaOH and 7.5 ml of purified heptane containing 1.5% isoamyl alcohol and was extracted for 20 min [10]. Heptane had been purified by successive washings of 1 N NaOH and 1N HCl and three washings with distilled water. After centrifugation for 5 min at 1000 rpm, 5 ml of the supernant was pipetted into 2.5 ml of 0.1 N HCl. After repeating the heptane extraction procedure, the contents of the tubes were shaken for 5 min and centrifuged for 5 min at 1000 rpm. The organic phase was removed by aspiration, and the acid phase was transferred to a glass scintillation vial. Ten ml of Triton X-100 scintillation cocktail was added, and radioactivity was measured by means of a Beckman LS-133 liquid scintillation counter. Counting efficiency was determined by external standardization. Nicotine content was expressed as dpm/g tissue.

Procedure

A total of 114 C57 and 114 DBA mice served as subjects in the experimental groups. Animals were pretreated with nicotine or saline three times a day at 4-hr intervals between 8 a.m. and 4 p.m. for 2, 4 or 7 days. On the next day (approximately 17 hr after the last pretreatment injection), each animal was given a challenge dose of nicotine or saline and tested for motor activity. Four treatment groups were thus constituted: nicotine pretreatment-nicotine challenge (NN), nicotine pretreatment-saline challenge (NS), saline pretreatment-nicotine challenge (SN), and saline pretreatment-saline challenge (SS). The NN treatment permitted observation of the development of tolerance, the NS treatment allowed an estimate of possible withdrawal reactions and of deleterious effects caused by repeated nicotine injections. The SN and SS treatments served as controls for possible unrecognized environmental variables and harmful effects of repeated injections.

Ten animals of both sexes in each strain were used for each 2 day pretreatment group, whereas n = 12 in the 4 day and n = 5 in the 7 day studies for each sex and strain. For the 2 day study these groups are designated 2NN, 2SN, 2NS and 2SS where 2 refers to the days of pretreatment, the first letter the type of treatment during these 2 days (nicotine, N or saline, S) and the second letter to the treatment preceding assessment of Y-maze activity. Only the NN treatment was used for the 4 and 7 day studies.

For comparison purposes, 10 C57 and 10 DBA mice of each sex received no pretreatment and were tested for motor activity 5 min after an injection of either nicotine or saline. These control groups are designated 0N (0 = no pretreatment, N = nicotine treatment before activity measurement) and 0S (S = saline treatment before activity measurement).

For all animals, liver and brain nicotine levels were determined 24 hr after activity measurement using the procedure described in the previous section.

Data Analysis

Since the variances of activity scores and of liver and brain nicotine levels for most groups were found to be non-homogeneous according to Hartley's F-Max test [6], the data were transformed to square root scores. This transformation satisfied the homogeneity of variance assumptions required for performing analysis of variance tests [26]. Analyses of variance were performed on all data, and these were followed by the Neuman-Keuls test of post-hoc comparisons to distinguish specific differences within significant main effects [13].

RESULTS

Nicotine (0N) and saline (0S) control groups were compared to NN, NS, SN and SS subgroups which were tested for motor activity on the day following 2 days of pretreatment. With respect to activity scores (Fig. 1), the main effect of treatment and all two-way and three-way interactions were statistically significant: treatment – $F(5,216) = 92.93$, $p<0.01$; treatment × strain – $F(5,216) = 6.48$, $p<0.05$; treatment × sex – $F(5,216- = 6.07$, $p<0.05$; treatment × strain × sex – $F(5,216) = 10.01$, $p<0.01$. These findings suggest a differential effect of treatment as a function of strain and sex. Application of the Neuman-Keuls test revealed that the 0N and 2NN treatments produced significantly different effects in DBA females ($p<0.05$), C57 males ($p<0.01$) and C57 females ($p<0.05$). Results of the 2NN and 2SN treatments were significantly different for these same strain and sex combinations

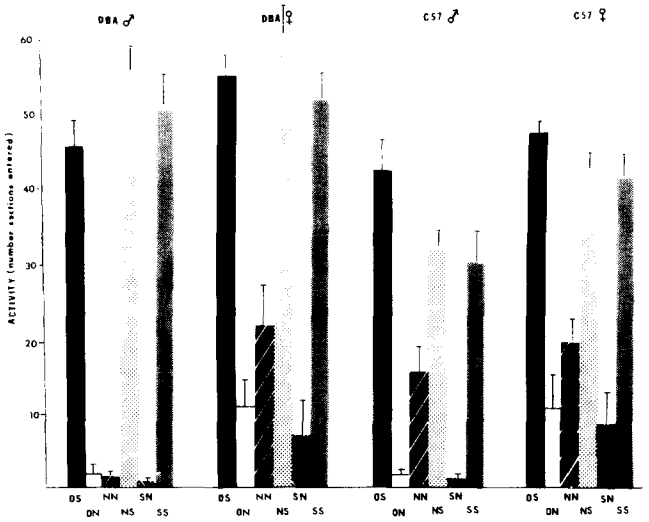

FIG. 1. Spontaneous motor activity in nicotine (0N) and saline (0S) control groups and in NN, NS, SN and SS subgroups tested after nicotine or saline challenge on the day following 2 days of pretreatment (mean + SE).

($p<0.01$ in each case). Thus, the development of tolerance to the depressant effects of nicotine on motor activity is suggested for all but the DBA males. The degree of tolerance development was highest in the C57 males, which showed an 86% increase in motor activity when scores of nicotine-pretreated animals were compared to nicotine control values. C57 females and DBA females demonstrated similar degrees of tolerance development, with 42% and 48% increases, respectively.

The 0S-2NS, 0S-2SS, 0N-2SN and 2NS-2SS comparisions revealed no differences that were statistically significant. Thus, neither multiple saline injections followed by saline or nicotine challenge nor multiple injections of nicotine following by saline challenge had any effect upon motor activity. This suggests that repeated injections produce no adverse effects upon motor behavior and that withdrawal from nicotine produces no change in this behavior when activity is measured 17 hr after the last injection.

Referring again to the data illustrated in Fig. 1, the main effect of strain and all two-way and three-way interactions were statistically significant: strain – $F(1,216) = 93.84$, $p<0.01$; strain × sex – $F(5,216) = 8.74$, $p<0.01$; other values as given above. The C57 animals had lower activity scores than the DBA's in all subgroups except 2NN males, 2SN males and 2SN females. For the 2NN treatment in males, the DBA scores were significantly lower than those for the C57's ($p<0.01$). This finding is consistent with the lack of tolerance development shown in DBA males by the 0N-2NN and 2NN-2SN comparisons.

The main effect of sex and all interactions were also statistically significant: sex – $F(1,216) = 4.88$, $p<0.05$; other values as given above. Males of each strain had lower activity scores than females for each of the six treatments. In the present study, DBA males showed significantly less activity than DBA females under the 2NN treatment condition ($p<0.05$). This difference points again to the lack of tolerance development in DBA males.

Figure 2 illustrates liver nicotine levels in nicotine (0N) and saline (0S) control animals in all 2xx subgroups. Treatment effects were not statistically significant for this measure, suggesting that multiple injections of nicotine or

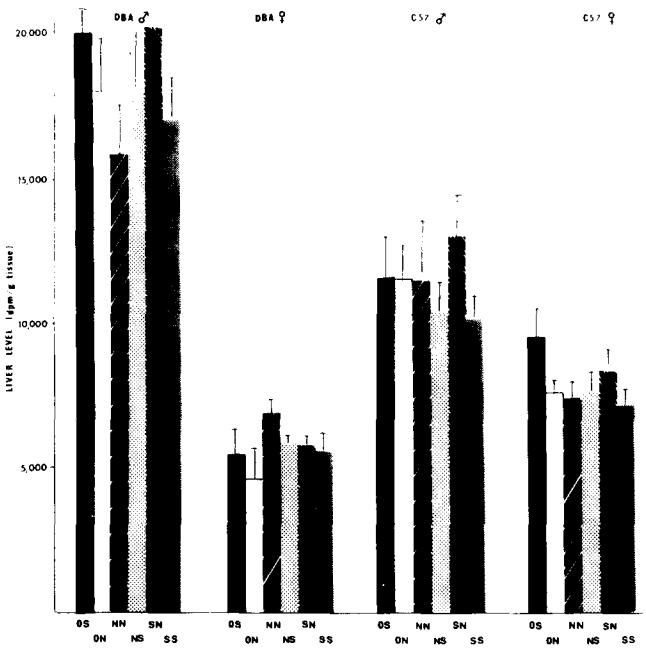

FIG. 2. Liver nicotine levels in nicotine (0N) and saline (0S) control groups and in NN, NS, SN and SS subgroups tested for motor activity on the day following 2 days of pretreatment (mean + SE).

saline for 2 days do not alter the rate of nicotine metabolism in the liver. That is, there is no development of metabolic tolerance. A significant effect of strain, $F(1,216) = 286.46$, $p<0.01$, and a significant strain × sex interaction, $F(5,216) = 61.90$, $p<0.01$, were found, indicating that strain and sex had differential influences on liver nicotine content. When all treatment groups were combined, C57 males showed 21% less liver nicotine than DBA males ($p<0.01$), and liver measures for DBA females were 20% lower than those for C57 females ($p<0.05$). The significant effect of sex, $F(1,216) = 4.93$, $p<0.05$, resulted from the fact that DBA females had 47% less liver nicotine than DBA males ($p<0.01$) and C57 females had 16% less than C57 males ($p<0.05$).

Brain nicotine levels in 0N, 0S and 2xx animals are shown in Fig. 3. The fact that treatment effects were not statistically significant suggests that multiple injections of nicotine for 2 days do not alter the level of nicotine in the brain. A statistically significant strain × sex interaction, $F(5,216) = 3.89$, $p<0.05$, indicated a differential effect of strain and sex on brain nicotine measures. The significant effect of sex, $F(1,216) = 3.96$, $p<0.05$, was caused by the finding of 22% less brain nicotine in DBA females than in DBA males ($p<0.01$). This difference is presumably attributable, at least in part, to the faster nicotine metabolism in the females.

Comparisons of 2NN, 4NN and 7NN Treatments

Activity levels and nicotine concentrations in liver and brain were compared for groups of animals that were challenged with nicotine on the day following 2, 4 or 7 days of pretreatment. (Since the comparisons described above demonstrated that repeated saline or nicotine injections had no adverse effects upon motor activity and did not alter liver or brain nicotine levels, only the NN treatment was used for animals pretreated for 4 or 7 days.)

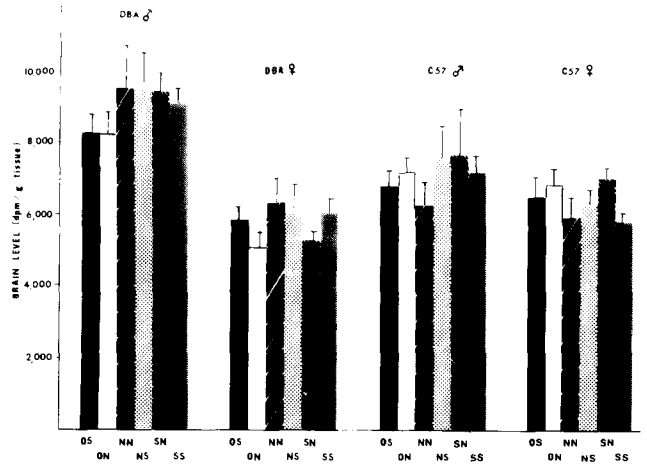

FIG. 3. Brain nicotine levels in nicotine (0N) and saline (0S) control groups and in NN, NS, SN and SS subgroups tested for motor activity on the day following 2 days of pretreatment (mean + SE).

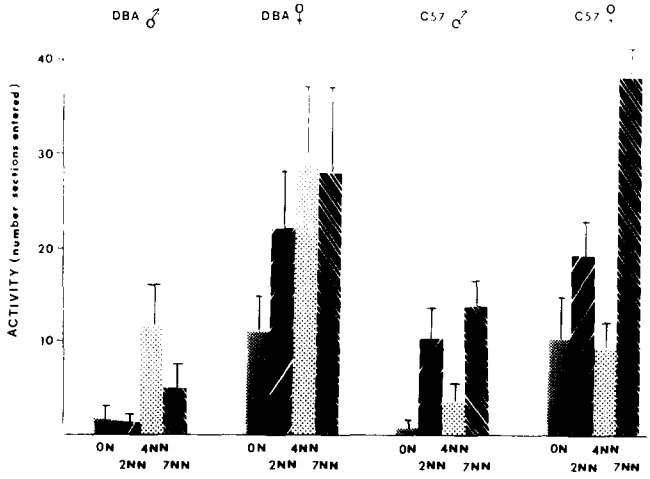

FIG. 4. Spontaneous motor activity in nicotine (0N) control groups and in subgroups tested after nicotine challenge on the day following pretreatment for 2 (2NN), 4 (4NN) or 7 (7NN) days (mean + SE).

With respect to the motor activity data illustrated in Fig. 4, the main effect of treatment and the treatment × strain were statistically significant: treatment – $F(2,98) = 3.49$, $p<0.05$; treatment × strain – $F(2,98) = 8.90$, $p<0.01$. This suggests that multiple injections of nicotine for 2, 4 or 7 days influence spontaneous motor activity differentially according to strain. In DBA animals, the activity scores of males were 88% higher for the 4NN treatment than for the 2NN ($p<0.05$), suggesting the development of behavioral tolerance when length of pretreatment was increased from 2–4 days. The slight increase in activity in DBA 2NN and 4NN females was not significant. The decrease between the 4NN and the 7NN treatment was not significant in either sex. In C57 animals, there was a slight decrease in activity between the 2NN and 4NN treatment, followed by a substantial and significant increase when length of pretreatment was increased from 4–7 days (76% increase in females, $p<0.01$; 73% increase in males, $p<0.05$). Thus, further development of behavioral tolerance is indicated in

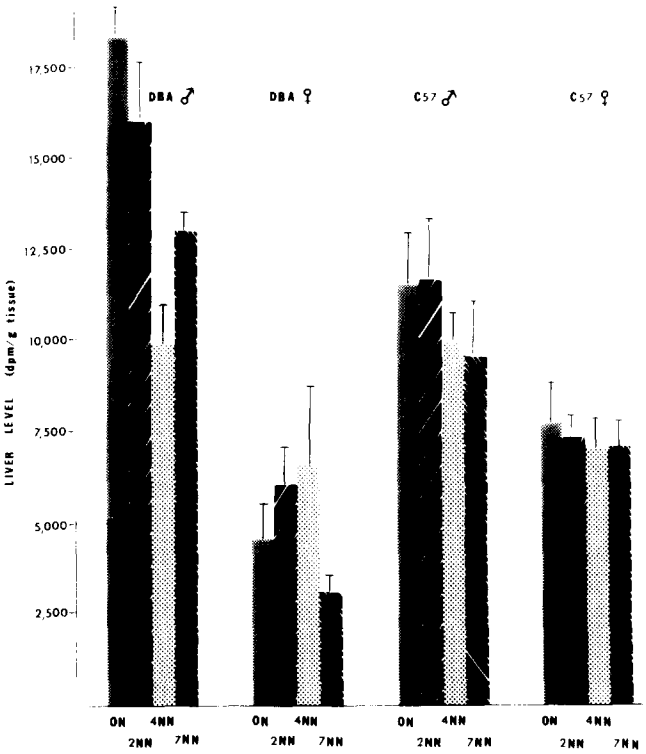

FIG. 5. Liver nicotine levels in nicotine (0N) control groups and in subgroups tested for motor activity after nicotine challenge on the day following pretreatment for 2 (2NN), 4 (4NN) or 7 (7NN) days (mean + SE).

both males and females, with a higher degree of tolerance development in females.

The main effect of sex was statistically significant, $F(1,98) = 36.18$, $p<0.01$. Males of each strain had lower activity scores than females for each of the treatments. In the DBA's, the differences were statistically significant for the 2NN, 4NN and 7NN treatments ($p<0.01$ in each case); C57 males and females differed significantly only after 7 days of pretreatment ($p<0.01$).

Figure 5 illustrates liver nicotine levels following 2, 4 or 7 days of pretreatment. The main effects of treatment and sex and the strain × sex interaction were statistically significant: treatment – $F(2,98) = 5.43$, $p<0.01$; sex – $F(1,98) = 99.88$, $p<0.01$; strain × sex – $F(1,98) = 23.90$, $p<0.01$. Liver measures in DBA animals showed a small (19%) decrease in nicotine content in males when length of pretreatment was increased from 2 to 4 days ($p<0.01$) and larger decreases in both males (55%) and females (30%) between the 2NN and the 7NN treatment ($p<0.05$ in females, $p<0.01$ in males). These findings suggest the development of drug dispositional tolerance in DBA animals. Females of each strain had lower liver nicotine levels than males for each of the treatments. DBA females exhibited 47% less liver nicotine than males for the 2NN treatment, 36% less for 4NN, and 51% less for 7NN ($p<0.01$ in each case). C57 females demonstrated significantly lower liver nicotine levels than males for the 2NN treatment (16%, $p<0.05$); the differences for 4NN and 7NN (16% and 13%, resepctively) were not statistically significant.

Brain nicotine levels following 2, 4 or 7 days of pretreatment are shown in Fig. 6. The measures did not

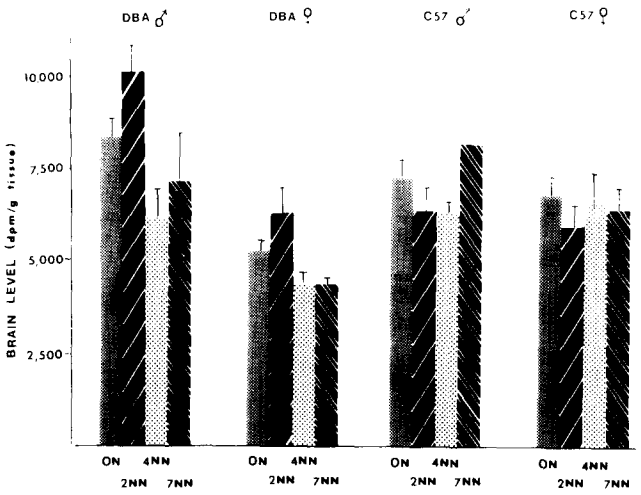

FIG. 6. Brain nicotine levels in nicotine (0N) control groups and in subgroups tested for motor activity after nicotine challenge on the day following pretreatment for 2 (2NN), 4 (4NN) or 7 (7NN) days (mean + SE).

differ significantly as a function of either treatment or strain. The main effect of sex and the treatment × strain and strain × sex interactions were statistically significant: sex – $F(1,98) = 18.45$, $p<0.01$; treatment × strain – $F(2,98) = 5.06$, $p<0.01$; strain × sex – $F(1,98) = 10.71$, $p<0.01$. DBA females had 23% less brain nicotine than males ($p<0.05$), while brain nicotine levels were nearly identical in C57 males and females.

DISCUSSION

When spontaneous motor activity in a Y-maze was measured after 2 days of nicotine pretreatment, the degree of behavioral tolerance development was twice as great in C57 males as in C57 or DBA females. However, C57 males had a slower liver metabolism rate and did not differ from either of the female groups in brain nicotine content. These findings suggest that there is a marked decrease in nervous system sensitivity to nicotine in C57 males after 2 days of repeated nicotine administration. Females of both strains also showed behavioral tolerance under this condition, with the degree of tolerance development being greater in the C57 animals. DBA males did not exhibit behavioral tolerance until length of pretreatment was increased to 4 days, while DBA females showed no greater tolerance development after 4 or 7 days of injections than after 2 days of pretreatment. Behavioral tolerance was demonstrated in both sexes of the C57 strain after 7 days of injections, with females showing a greater degree of tolerance development.

These results indicate that both genotype and sex influence the development of tolerance to nicotine's depressant effects on spontaneous motor activity. DBA males required the longest exposure to nicotine and C57 males required the shortest drug exposure for tolerance development. DBA and C57 females developed tolerance equally after 2 days of pretreatment, but the C57's showed a greater degree of tolerance after 7 days of injections than did the DBA's. The change in sensitivity to nicotine's motor effects after multiple injections can be rank ordered as follows: DBA males < C57 males < DBA females < C57 females.

The level of nicotine in the liver decreased significantly after 4 days of nicotine pretreatment in DBA males after 7 days in DBA females, suggesting the development of drug dispositional tolerance in DBA animals. C57 mice apparently did not develop drug dispositional tolerance, since both male and female liver nicotine levels were unaffected by repeated exposure to nicotine. Brain nicotine levels remained the same in all animals after repeated nicotine administration.

It should be noted that the 5 min time point used in these studies was in the linear portion, as measured on a semilog plot, of the nicotime disappearance curve for each of the four groups tested (unpublished data). As a result, liver nicotine levels at the 5 min time point should give a reliable estimate of relative nicotine metabolism rates. At the same time differences in behavioral effects of nicotine at 5 min should not be as influenced by differences in metabolism rate as these same effects would be at a later time point. Since brain nicotine levels were minimally altered by repeated administration of nicotine we can only assume that a significant portion of the decreased behavioral effects of nicotine seen at 5 min after injection is due to behavioral rather than drug dispositional tolerance.

The influence of genotype and sex on behavioral tolerance has also been shown in rats [22]. Female CD (Sprague-Dawley) and CDF (Fischer) rats developed a greater degree of tolerance to nicotine than males when spontaneous motor activity was measured in a Woodward activity cage. CDF females demonstrated greater tolerance development than CD females, while CD males showed a larger decrease in response to nicotine than CDF males. The change in sensitivity to nicotine's motor effects could be rank ordered as follows: CDF males < CD males < CD females < CDF females. Strain and sex differences in sensitivity to nicotine demonstrated no correlation with brain nicotine level. It can be concluded, therefore, that genotype and sex influence behavioral tolerance to nicotine in rats and mice and that no relationship between brain nicotine level and behavioral tolerance has been observed in either species.

Stolerman and his colleagues [23,24] found that the time interval between nicotine pretreatment and challenge affected development of behavioral tolerance in rats. When these investigators measured spontaneous motor activity in a Y-maze, they observed a progressive increase in tolerance to the challenge dose as the time interval between doses increased from 30 min to 2 hr. Maximum tolerance was exhibited at the 2-hr interval, and the effect had essentially disappeared after 8 hr. A comparison of these results with those of the present study suggests two interesting possibilities. First, since we observed behavioral tolerance (measured in the same manner as in Stolerman's research) 17 hr after the last nicotine pretreatment, it may be that mice retain tolerance for a longer time than do rats. Because changes in nervous system sensitivity appear to be involved in nicotine tolerance, this suggests that species comparisons between mice and rats might show interesting differences in number of brain nicotine receptors and/or in receptor affinity for nicotine. Second, it appears that the effects of genotype and sex on tolerance development observed in the present study might have varied if alternative time intervals between pretreatment and challenge had been used. For example, the degree of tolerance demonstrated under these

conditions might be maximal for mice of a particular strain and sex, but less than maximal for other strain-sex combinations.

It has also been shown [23,24] that the dose of nicotine used to pretreat rats can be a critical factor in determining the magnitude of tolerance that develops. It seems that there is an optimal dose for eliciting tolerance and that larger or smaller doses are less effective. In addition, the challenge dose has been shown to affect sensitivity to nicotine. If too high a dose is used before testing, tolerant rats show complete suppression of spontaneous motor activity. Thus, the effect of the challenge dose itself may lead to the conclusion that tolerance is weak or absent.

In view of these findings, it should be noted that the demonstrated effects of genotype and sex on the development of behavioral tolerance to nicotine in mice may be specific to the particular interval between pretreatment and challenge and the particular doses used in the present study. We intend to explore this possibility in future experiments using other nicotine doses and schedules of testing.

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