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ALPHA-METHYL-PARA-TYROSINE EFFECTS IN MICE SELECTIVELY BRED FOR DIFFERENCES IN SENSITIVITY TO ETHANOL

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Abstract—The responses of catecholamine systems in long sleep (LS) and short sleep (SS) mice to α-methyl-*p*-tyrosine (AMPT) have been examined. Marked differences were found between LS and SS mice in the dose necessary for maximal brain catecholamine depletion and in the time-course of the catecholamine depletion. Brain catecholamines in the LS mice were depleted by lower doses of AMPT and the levels remained depressed for longer periods of time in this line of mice. These differences may be explained only partially by an increased susceptibility of the LS mice to the hypothermia and toxic effects caused by AMPT administration, as they persist with non-toxic AMPT dosage regimens and under conditions where the degree of hypothermia is comparable in both lines of mice. In addition, there were no differences between the K_i values for the effect of AMPT on the tyrosine hydroxylase from striata of these mouse lines. The primary cause of the heightened response to AMPT in LS mice would appear to be pharmacokinetic in nature, as brain and plasma peak levels of AMPT in LS mice were greater and the levels remained higher for a longer time. The depletion of brain tyrosine by AMPT combined with the lower affinity of the LS striatal tyrosine hydroxylase for the substrate tyrosine may also contribute to the heightened response in LS mice.

McClearn and Kakihana [1] have, through a selective breeding program, established two lines of mice that differ in their sensitivities to a hypnotic dose of ethanol. These lines of mice are designated long sleep (LS) and short sleep (SS). After a 4.0 g/kg dose of ethanol, the present generations lose their righting reflex ("sleep") for an average of 13 min (SS) and 120 min (LS). Previous studies have demonstrated that the difference in ethanol-induced sleep times is primarily due to a difference in the CNS sensitivity to ethanol [2, 3].

A variety of genetic differences between the LS and SS mice have been reported, including a greater increase in locomotor activity in SS mice after low doses of ethanol or pentobarbital [4], more severe withdrawal reactions in SS mice after chronic ethanol administration [5], more severe hypothermia in LS mice after ethanol administration [6], greater free choice ethanol consumption in SS mice [7], greater rise in plasma corticosterone levels in LS mice after ethanol administration [8], and a greater sensitivity of LS cerebellar Purkinje cells to applied ethanol [9, 10].

In addition, there are a number of reports that indicate specific differences in central catecholaminergic systems in LS and SS mice, including longer loss of righting reflex in LS mice after salsolinol [11] and gamma-butyrolactone treatment [12], greater decrease in dopamine turnover in LS mice after ethanol administration [13], lower density of β-adrenergic receptors in LS mice in cerebral cortex [14], more rapid activation of tyrosine hydroxylase (TH) in the hypothalamus of SS mice after ethanol [15], increase in SS mice and decrease in LS mice in the ethanol-induced sleep times after intracerebro-ventricular injections of catecholamine agonists [16], a greater depressant effect of ethanol on the *in vivo* tyrosine hydroxylation rate in the cerebellum, locus ceruleus, hypothalamus and frontal cortex of LS mice [17], and a differential activation of adrenal gland TH in SS mice after ethanol [18]. These results suggest that intrinsic differences in catecholamine systems in the LS and SS mice may be, at least in part, responsible for the difference in CNS sensitivity to ethanol.

The present study was designed as an extension of earlier experiments in which it was observed that reserpine (1–5 mg/kg, i.p.) produces a dose-dependent decrease in ethanol-induced sleep times in the LS mice after 10 days, while the sleep times are increased after reserpine in the SS mice [16, †]. Since reserpine depletes tissue stores of both catecholamines and indoleamines, it seemed appropriate to assess the effects of a more selective depletor of catecholamine stores, α-methyl-*p*-tyrosine (AMPT). AMPT is a competitive inhibitor of TH, the rate-limiting enzyme in the biosynthetic pathway for catecholamines [19]. The drug has been used extensively to determine the functional roles of catecholamines in brain and to estimate the turnover of catecholamines in various tissues (for review, see Ref. 20). We report here that the effects of AMPT differ considerably in LS and SS mice even in the absence of administered ethanol.

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† T. A. French and N. Weiner, manuscript in preparation.

MATERIALS AND METHODS

Drugs. (−)-Norepinephrine bitartrate, dopamine hydrochloride, dihydroxybenzylamine hydrobromide and DL-α-methyl-*p*-tyrosine methyl ester (AMPT) were purchased from the Sigma Chemical Co. (St. Louis, MO). Other chemicals were purchased from the following companies: 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropteridine HCl (6-MePtH$_4$) (Calbiochem, San Diego, CA); catalase (Boehringer-Ingelheim, Ltd., Elmsford, NY); L-[1-^{14}C]tyrosine (New England Nuclear, Boston, MA); NCS solubilizer (Amersham Searle Corp., Arlington Heights, IL); L-ascorbic acid (J. T. Baker Chemical Co., Phillipsburg, NJ); pyridoxal phosphate and 3-iodotyrosine (Sigma Chemical Co.). All other reagents were of the highest purity commercially available.

Animals. Male and female LS and SS mice were obtained from the Institute of Behavioral Genetics, University of Colorado, Boulder, CO. The mice were housed fifteen per cage, according to sex, and allowed free access to food and water. The mice were of the 32nd generation and were used at 50–80 days of age.

Drug treatments and sleep times. The methyl ester of AMPT was administered intraperitoneally in saline in a volume of 10 ml/kg. Sleep times were measured in mice who had received either saline or AMPT (60 mg/kg) every 4 hr for a total of three doses. Four hours after the last saline or AMPT injection, ethanol was administered (4.0 g/kg, i.p.). Sleep times were recorded as the time after ethanol injection when the mice lost their righting reflex until the mice were able to right themselves at least three times within 1 min.

Tissue preparation and assay of TH. Kinetic studies of TH were performed on striata of untreated mice. The mice were decapitated and the brains removed and maintained on ice at 4°. The striata were dissected out [21] and immediately frozen on dry ice for assay of TH activity. The tissue was weighed and homogenized in 15 vol. of 0.05 M Tris-acetate buffer (pH 6.2) containing 20 mM sodium fluoride and 0.2% Triton X-100. The homogenates were centrifuged at 40,000 *g* for 30 min at 4°.

The TH activity of the resulting striatal supernatant fraction was determined by means of the coupled decarboxylase assay [22, 23] as modified in our laboratory. The standard assay mixture (25 μl) contained 120 mM *N*-tris(hydroxy-methyl)methyl-2-aminoethanesulfonic acid (TES) buffer (pH 6.3), 1300 units of catalase, 5 mM ascorbic acid, 2.0 mM 6-MePtH$_4$, 0.2 mM [1-^{14}C]L-tyrosine (sp. act. 58 mCi/mmole) and 10 μl of tissue. Six concentrations of tyrosine ranging from 0.001 to 0.2 mM were used for the determination of the K_m of TH with respect to tyrosine. To determine the K_i for AMPT, six concentrations of AMPT from 0.001 to 0.5 mM were used.

The reaction was initiated by placing the tubes in a 37° bath. Samples were incubated for 20 min and then transferred to an ice bath. The enzyme reaction was determined to be linear with enzyme concentration and time. At the end of the incubation, 10 μl of decarboxylation mix containing 3.9 mM 3-iodotyrosine, 1.5 mM pyridoxal 5′-phosphate, 300 mM bis-Tris buffer (pH 9.8), and 3 μl of hog kidney L-aromatic amino acid decarboxylase was added. The tubes were capped with a rubber septum from which a plastic well containing 100 μl of NCS solubilizer was suspended. The assay tubes were then incubated for an additional 30 min at 37° to allow for the quantitative conversion of [^{14}C]dopa to $^{14}CO_2$ and dopamine. The reaction was stopped by injecting 0.1 ml of 1.6 N perchloric acid into the assay tube, and the tubes were incubated for an additional hour at 37° to allow for quantitative collection of the liberated CO_2. Radioactivity was determined by liquid scintillation spectrometry. Protein was determined by the method of Lowry *et al.* [24]. Results are expressed as nanomoles of product formed per milligram protein per hour.

Catecholamine levels. Whole brains, including brain stem and cerebellum, were used for analysis. The brain was weighed and homogenized in 3.0 ml of 0.1 M perchloric acid containing 50 ng of dihydroxybenzylamine as an internal standard. The homogenate was centrifuged at 28,000 *g* for 30 min at 4° and 2 ml of the resulting supernatant fraction was combined with 5 ml of 40 mM sodium phosphate (pH 7) and 0.2 ml of 3 M Tris–HCl buffer (pH 8.6) to bring the final pH to 8.5. The solution was passed through a column containing 200 mg of acid-washed alumina, the alumina was washed with 10 ml of distilled water, and catechols were eluted with 2 ml of mobile phase (pH 2.85). Mobile phase contained 0.2 M phosphoric acid, 0.05 M trichloroacetic acid, 2% methanol and 0.2 M EDTA. An aliquot of the mobile phase eluate was injected into the high-pressure liquid chromatographic system for analysis of norepinephrine and dopamine.

The high-pressure liquid chromatographic system consisted of two Altex model 100A dual piston pumps equipped with pulse dampener, an electrochemical detector (LC-3 amperometric detector, Bioanalytical Systems, Inc., West Lafayette, IN) and a Lichrosorb RP-18, 10 μm column, 250 mm (length) × 4.6 mm (i.d.) (E. Merck, Darmstadt, West Germany). The detector electrode was set at a fixed potential of + 0.6 V. The flow rate was 1.25 ml/min and the sample volume 100 μl.

Concentrations of catecholamines in brain were calculated from values obtained from a standard curve for known amounts of norepinephrine and dopamine treated in a similar manner. Catecholamine levels were calculated by a peak height analysis relative to the internal standard in the homogenization solution. Data are presented as nanograms of catecholamine per milligram of tissue, wet weight.

Temperature measurement. Rectal body temperature was measured with a YSI model 44TA thermometer (Yellow Springs Instrument Co., Yellow Springs, OH). The rectal probe was lubricated with glycerol and inserted 1.5 cm into the rectum. Body temperature was determined over an 18-hr interval following AMPT administration (250 mg/kg, i.p.) to mice housed at either 20° or 28°.

Measurement of AMPT and tyrosine. AMPT and tyrosine were measured by gas chromatography/mass spectrometry, employing a stable isotopically

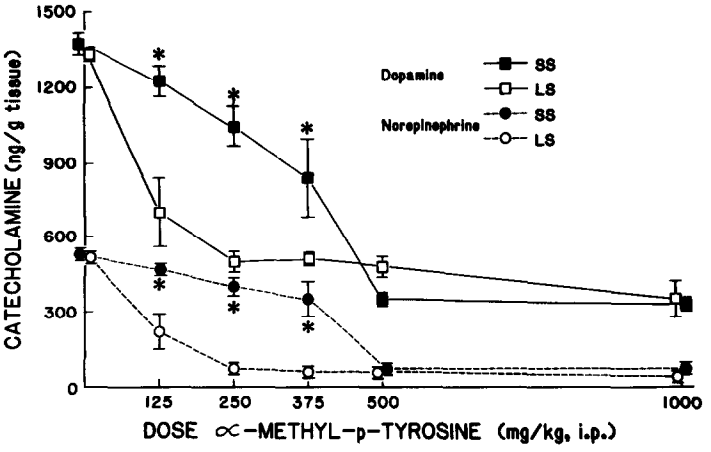

Fig. 1. Dose–response relationship between brain catecholamine levels following administration of AMPT. Different doses of AMPT were administered intraperitoneally to long sleep (LS) and short sleep (SS) mice, and brain catecholamine levels were measured 18 hr later. Each value represents the mean value (± S.E.) from five or six individual mice. Key: (*) significantly different from long sleep, $P < 0.05$.

labeled internal standard. Oxygen-18 labeled AMPT and tyrosine were synthesized by acid catalyzed exchange of the two carboxyl oxygen atoms with HCl in oxygen-18 water [25] and purified by recrystallization. The standard was added to either mouse brains or plasma and the brains were then homogenized and the amino acids from brain or plasma were isolated by the method of Clay and Murphy [26]. Gas chromatography was at 180° isothermal with a column of 3% OV-101. Ions selected for monitoring were masses 232 and 236 for AMPT and its ^{18}O-analogue, respectively, and masses 218 and 222 for tyrosine and its ^{18}O-analogue. Conversion of the isotope ratios to micrograms of AMPT or tyrosine in the brain or plasma was performed by linear regression analysis of a standard curve generated simultaneously with the brain or plasma samples.

Calculations and statistics. All differences between treatment groups were analyzed by Student's *t*-test for unpaired samples. For the kinetic studies, the results were analyzed according to the method of Lineweaver and Burk (primary plot of reciprocal velocity versus the reciprocal concentration of substrate in the absence and presence of inhibitor), and the kinetic constants were determined by linear regression analysis. The K_i was obtained by determination of the slope of enzyme activity versus inhibitor concentration [27].

RESULTS

Effect of AMPT dosage on catecholamine levels. Figure 1 illustrates the effect of various doses of AMPT on LS and SS brain norepinephrine and dopamine levels measured 18 hr after intraperitoneal administration. Eighteen hours was chosen since Corrodi and Hanson [28] had observed a 75–80% depletion of brain norepinephrine and dopamine in mice 16–20 hr after a single dose of 250 mg/kg of AMPT. There appears to be a much greater inhibition of catecholamine synthesis in the LS mice after administration of 125, 250 and 375 mg/kg of AMPT, as suggested by the greater decline in catecholamine levels with these doses (Fig. 1). At doses of 500 and 1000 mg/kg, the AMPT-induced depletion of catecholamines at 18 hr was comparable in the SS and LS mice.

Time-course of catecholamine depletion and recovery after AMPT. To determine whether the difference in catecholamine levels between LS and SS mice 18 hr after AMPT is due to a greater resistance of SS mice to inhibition of synthesis or a shorter duration of the action of this substance in SS mice, a time-course of the effects of AMPT on brain catecholamine levels was performed. The initial rate of catecholamine depletion after administration of 250 mg/kg of AMPT indicates that the inhibition of catecholamine synthesis in the first 3 hr was comparable in the two lines of mice (Fig. 2). After this time, inhibition of catecholamine synthesis in the LS mice apparently persisted for at least 18 hr while in SS mice the effect of AMPT appeared to diminish after 3–6 hr.

Since 10% (2 of 21) of the SS mice and 18% (4 of 22) of the LS mice died during the first 18 hr after injection of 250 mg/kg AMPT, the differential response seen after AMPT may be due to a nonspecific toxic action of the drug, such as renal damage [29, 30], rather than a selective action of AMPT at the catecholaminergic neuron. However, the catecholamine levels in the brains of LS mice returned to control values by 24–48 hr (Fig. 2). Thus, the differential response to AMPT is probably not due to a selective toxicity in the LS mice that is irreversible or that persists for longer than 1–2 days.

Effect of low-dose AMPT on catecholamine levels and sleep time. To eliminate possible complications due to the toxicity of AMPT, a dose and route of administration that are not lethal and do not cause demonstrable toxicity were selected. Rech *et al.* [31] reported that animals given multiple intraperitoneal injections of AMPT (50 mg/kg × 3) behave very

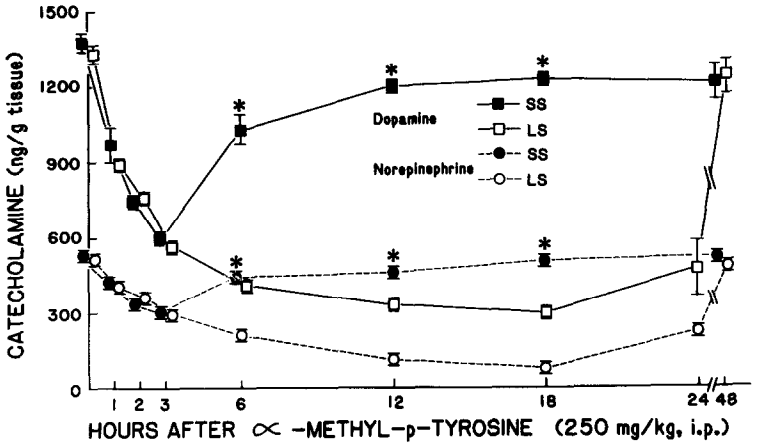

Fig. 2. Time-course of brain catecholamine levels after AMPT. Long sleep (LS) and short sleep (SS) mice were treated with AMPT (250 mg/kg, i.p.), and brain catecholamine levels were measured over a 48 hr period. Each value represents the mean value (± S.E.) from five or six individual mice. Key: (*) significantly different from long sleep, $P < 0.05$.

similarly to untreated normal animals except for slight ptosis and loss of skeletal muscle tone, even though the degree of catecholamine depletion is comparable to that seen after a single 200 mg/kg dose given intraperitoneally (less than 20% of control). LS and SS mice were given i.p. injections of 60 mg/kg of AMPT every 4 hr for three doses and brain catecholamines were measured every 4 hr (Fig. 3). Although the depletion of catecholamines seen with this dosage regimen was not as great as with a single 250 mg/kg, i.p. injection, a significant depletion of norepinephrine and dopamine in both lines of mice was demonstrable 4 hr after the last injection. Norepinephrine levels are 54% (LS) and 70% (SS) of control, while dopamine levels are 52% (LS) and 64% (SS) of control. It is important to note that, in the absence of any obvious AMPT-induced toxicity (no behavioral effects were noted and the substance was not lethal to the mice in this dosage regimen), there was still a greater depletion of brain catecholamines at all times measured in LS mice. However, the difference was not as great as that seen with a single 250 mg/kg, i.p. injection.

† T. A. French and N. Weiner, manuscript in preparation.

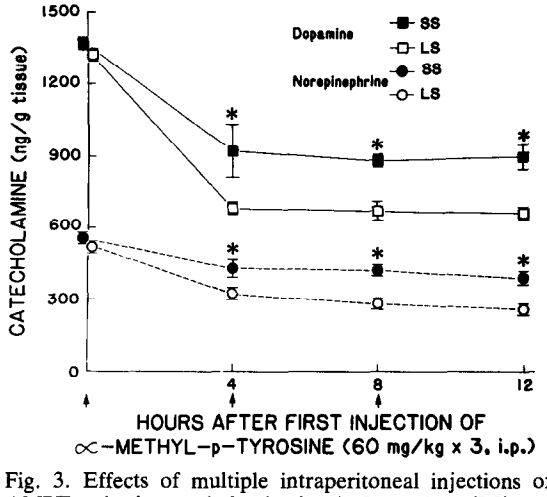

Fig. 3. Effects of multiple intraperitoneal injections of AMPT on brain catecholamine levels. Long sleep (LS) and short sleep (SS) mice were treated with 60 mg/kg of AMPT every 4 hr (↑) for a total of one, two, or three doses. Four hours after the first, second, or third injections, brain catecholamine levels were measured. Each value represents the mean value (± S.E.) from four individual mice. Key: (*) significantly different from long sleep, $P < 0.05$.

Since no observable toxicities were associated with this dosage regimen (60 mg/kg × 3), it was possible to measure the effect of the catecholamine depletion on the ethanol-induced sleep times. In the LS mice the sleep times were reduced from 135 to 120 min ($P < 0.05$) while the sleep times in the SS mice were increased from 20 to 29 min ($P < 0.05$). These changes in sleep times are in the same direction as seen previously after 6-hydroxydopamine and reserpine† administration, although they were not as pronounced. The smaller effect on sleep times may be due to the less complete catecholamine depletion seen after this regimen of AMPT administration.

Effect of AMPT on body temperature. The hypothermic responses induced by both ethanol [32] and reserpine† appear to be different in the two lines of mice. The ethanol-induced hypothermia is more severe in LS mice, whereas reserpine-induced hypothermia is greater in SS mice. The greater degree of hypothermia following reserpine administration, that was produced in the SS mice, may have impaired their ability to replenish catecholamine stores after they were depleted.† For these reasons the body temperature of the LS and SS mice was monitored over the first 18 hr after AMPT administration (250 mg/kg, i.p.). When the experiment was performed at room temperature (20°), the body temperature of the LS mice fell to a significantly lower level than that in the SS mice at 18 hr, 31.1 ± 1.6° compared to 36.1 ± 1.0° (Fig. 4). These values are

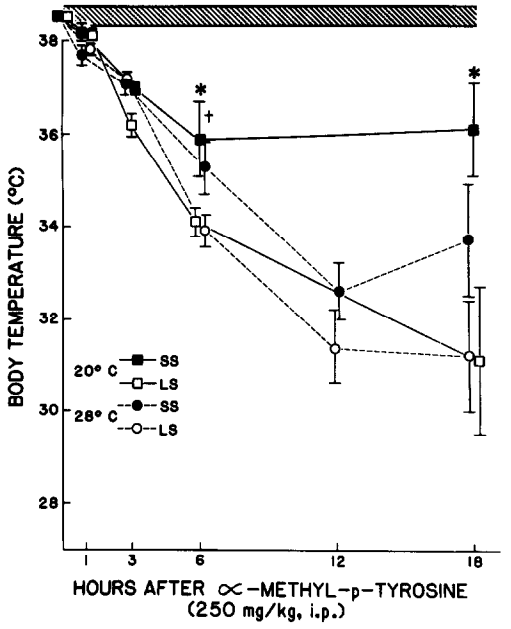

Fig. 4. Effect of environmental temperature on AMPT-induced hypothermia. Long sleep (LS) and short sleep (SS) mice were treated with AMPT (250 mg/kg, i.p.), and rectal temperature was monitored for 18 hr under two environmental conditions. One group (squares) was housed at ambient temperature (20°) and another group (circles) was housed at an elevated temperature (28°). Each value represents the mean value (± S.E.) from four to six individual mice. Key: (*) significantly different from long sleep (20° group), $P < 0.05$; and (†) significantly different from long sleep (28° group), $P < 0.05$.

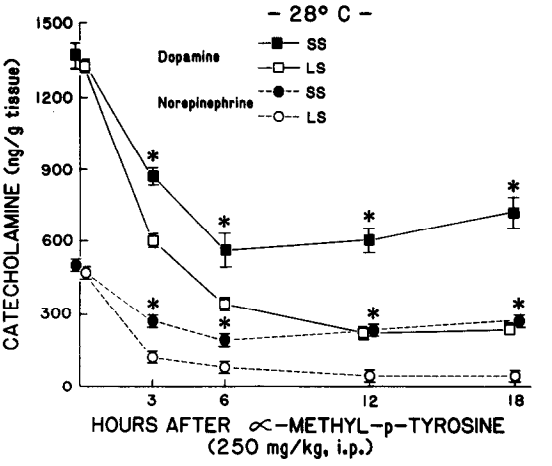

Fig. 5. Effect of environmental temperature on brain catecholamine levels after administration of AMPT. Long sleep (LS) and short sleep (SS) mice were treated with AMPT (250 mg/kg, i.p.) and housed in a heated environment (28°). Catecholamine levels were measured over an 18-hr period. Each value represents the mean value (± S.E.) from four individual mice. Key: (*) significantly different from long sleep, $P < 0.05$.

very similar to those obtained by Moore *et al.* [29] in rats that were judged to be either "affected" (characterized by emaciation, lethargy, piloerection and hypothermia) or "non-affected" (no observable symptoms) by AMPT (200–300 mg/kg, i.p.). It is therefore possible that the large differences in the duration of catecholamine synthesis inhibition between LS and SS mice were secondary to the differences in the decline in body temperature.

In the reserpine studies,‡ maintaining the mice in an environment with an elevated temperature eliminated the difference between the hypothermic responses of the SS and LS mice. The ability of the SS mice to replenish catecholamine stores also was enhanced at the elevated environmental temperature following reserpine administration although it remained significantly less than that of the LS mice. When the mice were housed in a 28° environment following AMPT administration, the temperature reponse was essentially the same in both lines of mice (Fig. 4). The brain catecholamine levels were measured during this 18-hr period and, even though the differences in catecholamine levels were not as great as when the mice were housed at room temperature, catecholamine levels were still 3- to 5-fold higher in the SS mice by 18 hr (Fig. 5). It would appear that the difference in the degree of hypothermia between the LS and SS mice (Fig. 4) cannot explain entirely the difference in catecholamine depletion following AMPT administration (Fig. 2).

Tyrosine hydroxylase kinetics. The time-course of catecholamine depletion following AMPT suggests that a difference may exist in the interaction of the competitive inhibitor AMPT with TH in the LS and SS mice. The *in vitro* kinetics of TH from the striatum of LS and SS mice have been analyzed both in the presence and absence of AMPT in order to determine whether there is an inherent difference in the interaction of enzyme and inhibitor in the two lines of mice (Table 1). Tyrosine was used as the variable substrate, and the pterin cofactor was held constant at saturating levels (2 mM) in the assay. No difference was apparent in the V_{max} of striatal tyrosine hydroxylase in the two lines, but the apparent K_m for tyrosine was slightly lower in the SS mice (0.07 compared to 0.11 mM), indicative of a greater affinity of the SS striatal enzyme for the tyrosine substrate. The K_i was determined for AMPT versus the tyrosine substrate, and there was no difference between the enzyme from LS or SS mice. The K_i for AMPT was approximately 25 μM in both lines of mice.

Brain levels of AMPT. The lack of a difference between LS and SS mice in the interaction of enzyme and inhibitor raises the possibility that a pharmacokinetic difference could account for the differential response to AMPT. A 250 mg/kg dose of AMPT was administered to both lines of mice and the whole brain levels of AMPT were measured during the ensuing 12 hr. At 1, 3, and 6 hr, the brain levels of AMPT were significantly higher in the LS mice (Fig. 6). Thus, the AMPT is apparently not being accumulated in the brains of SS mice to as great a degree as in LS mice, nor does it persist as

‡ T. A. French and N. Weiner, manuscript in preparation.

Table 1. Kinetic parameters of striatal tyrosine hydroxylase*

	V_{max} (nmoles/hr/mg protein)	K_m (mM)	K_i (μM)
Long sleep	16 ± 2	0.11 ± 0.01	25 ± 7
Short sleep	15 ± 1	0.07 ± 0.01†	27 ± 8

* Each value in the table is the mean of four experiments ± S.E. The V_{max} and K_m were determined with respect to the tyrosine substrate, which was employed in six concentrations ranging from 0.001 to 0.2 mM. The 6-methyltetrahydropterin cofactor was held constant at 2.0 mM. The K_i for AMPT was determined as described in Materials and Methods with six concentrations of AMPT ranging from 1 to 500 μM.

† Significantly different from long sleep, $P < 0.01$

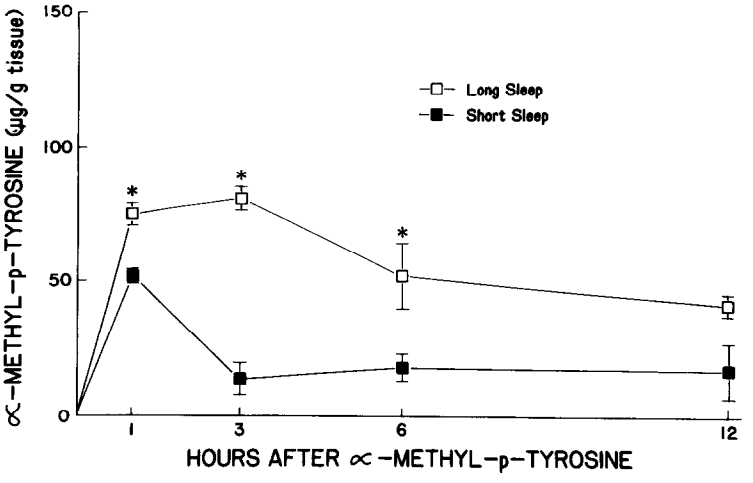

Fig. 6. Brain levels of AMPT at 1, 3, 6 and 12 hr after administration of AMPT (250 mg/kg, i.p.). Long and short sleep mice were treated with AMPT, and the brain levels of AMPT were measured over a 12-hr period. Each value represents the mean value (± S.E.) from three to six individual mice. Key: (*) significantly different from short sleep, $P < 0.05$.

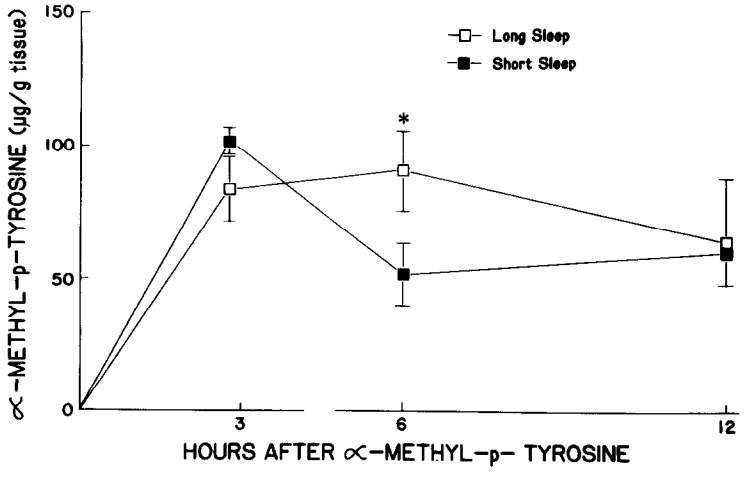

Fig. 7. Brain levels of AMPT at 3, 6, and 12 hr after administration of AMPT (500 mg/kg, i.p.). Each value represents the mean value (± S.E.) from three or four individual mice. Key: (*) significantly different from short sleep, $P < 0.05$.

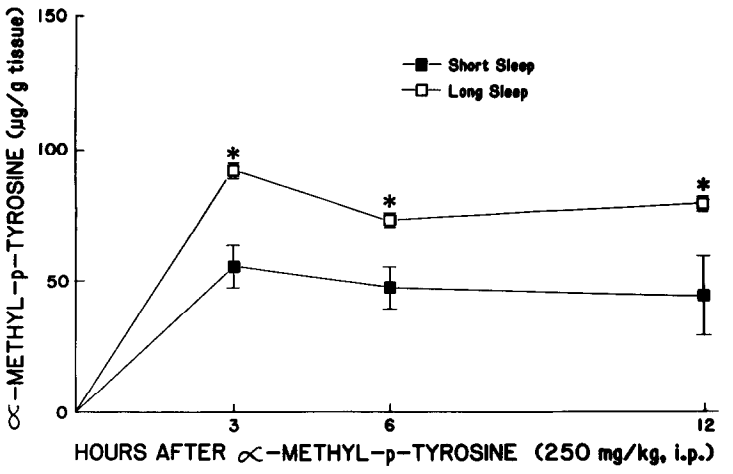

Fig. 8. Brain levels of AMPT at 3, 6, and 12 hr after administration of AMPT (250 mg/kg, i.p.) in mice housed in a 28° environment. Each value represents the mean value (± S.E.) from three individual mice. Key: (*) significantly different from short sleep, $P < 0.05$.

long in the brains of the SS mice. This difference in the brain levels of AMPT is apparently dose dependent as shown by the time-course of brain AMPT concentrations following a dose of AMPT of 500 mg/kg (Fig. 7). This higher dose of AMPT, which produced the same level of catecholamine depletion in LS and SS mice (Fig. 1), produced comparable levels of AMPT in the brains of the two lines of mice at 3, 6, and 12 hr. The brain levels of AMPT (250 mg/kg) were also measured in mice that were housed at an elevated environmental temperature (Fig. 8). The difference in levels, although not as great as that seen in mice housed at room temperature, was still evident, as was the difference in catecholamine depletion between LS and SS mice (Fig. 5).

Plasma levels of AMPT. The levels of AMPT in plasma at 1, 3, 6 and 12 hr after administration of a 250 mg/kg dose of AMPT were also ascertained (Fig. 9). Plasma levels of AMPT in the LS mice were significantly higher than the levels in SS mice at 1, 3 and 6 hr. These results are similar to those seen when brain levels of AMPT were measured at these time intervals (Fig. 6).

Effect of AMPT on brain tyrosine levels. The effect of AMPT (250 mg/kg) on brain levels of the amino acid precursor for catecholamine synthesis, tyrosine, was also determined. Brain tyrosine levels were obtained at 1, 3, 6 and 12 hr after AMPT administration (Fig. 10). Brain tyrosine levels were reduced by AMPT treatment, reaching a nadir by 6 hr and returning to near control levels by 12 hr. The degree of the reduction in brain tyrosine levels in the two lines of mice was not significantly different.

DISCUSSION

The present results demonstrate a very pronounced difference between the LS and SS mice in

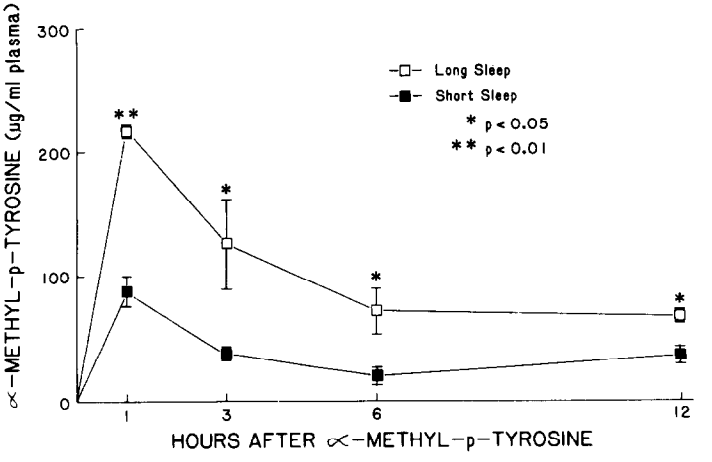

Fig. 9. Plasma levels of AMPT at 1, 3, 6 and 12 hours after administration of AMPT (250 mg/kg, i.p.). Each value represents the mean value (± S.E.) from four or five individual mice. Key: (**) significantly different from short sleep, $P < 0.01$; and (*) significantly different from short sleep, $P < 0.05$.

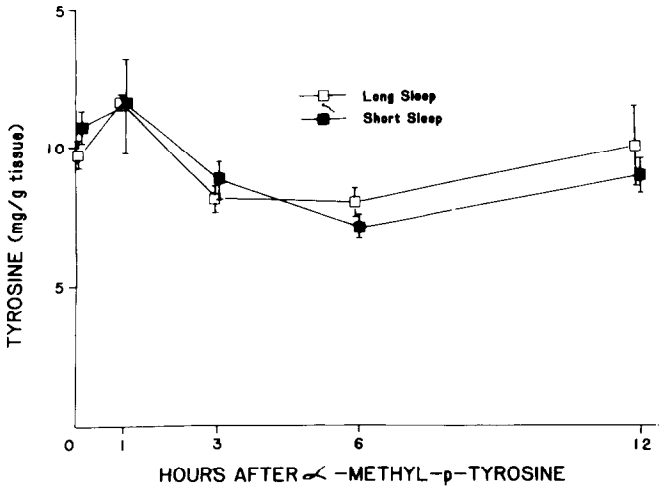

Fig. 10. Effect of AMPT (250 mg/kg, i.p.) on brain tyrosine levels. Long sleep (LS) and short sleep (SS) mice were treated with AMPT, and the brain levels of tyrosine were measured over a 12-hr period. Each value represents the mean value (± S.E.) from three to twelve individual mice.

the depletion of brain catecholamines following an intraperitoneal injection of AMPT. This difference is reflected in both the AMPT dose–response curve and the time-course of brain catecholamine depletion following 250 mg/kg of AMPT. The greatest difference between the lines was seen with a dose of 250 mg/kg, the same dose that has been used to determine brain catecholamine turnover in these lines of mice [13]. Eighteen hours following administration of this dose of AMPT, norepinephrine and dopamine levels in brains of LS mice were 14 and 38% of control, respectively, while in the SS mice the whole brain levels of norepinephrine and dopamine were 75 and 76% of control respectively. The 250 mg/kg dose of AMPT had also been used previously by Corrodi and Hanson [28], who reported that catecholamine levels in albino mice decline slowly and reach a minimum of 20–25% of control values after 16–20 hr. This is similar to what was observed in the present study in the LS mice 18 hr following AMPT administration. Thus, the SS mice are apparently less susceptible to catecholamine synthesis inhibition by AMPT than either of these strains of mice. It was only after a dose of at least 500 mg/kg of AMPT that the brain catecholamines were depleted to a level at 18 hr in SS mice which was comparable to that obtained in LS mice with either this dose or the 250 mg/kg dose.

The time-course of brain catecholamine depletion and recovery following 250 mg/kg of AMPT indicates that AMPT inhibited catecholamine synthesis to the same extent during the first 3 hr in both lines of mice. Semilogarithmic plots of catecholamine concentration versus time for the first 3 hr are linear and the turnover rates have been calculated from the slopes of these plots. Turnover rates determined in this manner were similar in LS and SS mice for both norepinephrine and dopamine. For norepinephrine the rates were 1.19 ng/g/hr (LS) and 1.20 ng/g/hr (SS). The dopamine turnover rates were 1.33 ng/g/hr (LS) and 1.30 ng/g/hr (SS). These rates are comparable to those reported by Collins *et al.* [13] for the LS mice (norepinephrine, 1.23; dopamine, 1.35) but are lower than those reported for the SS mice (norepinephrine, 1.46; dopamine, 1.58).

The difference in the responses of LS and SS mice to AMPT was apparent only after the first 3 hr. At 6 hr after 250 mg/kg of AMPT the catecholamine levels in SS mice had begun to return toward control levels and by 18 hr the levels were at 95% of control values. In contrast, catecholamine levels in LS mice continued to decline after 3 hr, reaching a minimum of 13% of control values by 18 hr. Doses of AMPT greater than 100 mg/kg administered intraperitoneally have been reported to cause renal lesions and even death in rats [29, 30]. In the present experiments, in the first 18 hr after AMPT a slightly greater proportion of the LS mice (4 of 22) died than in the SS group (2 of 21). However, by 24 hr after AMPT, brain catecholamine levels in the surviving LS mice began to recover and by 48 hr the catecholamine levels had returned to control values. Since the brain catecholamine levels in the LS mice eventually recovered, and the mortality rate in the LS mice was not markedly greater than in SS mice, it would appear that the difference in response of catecholaminergic systems in the two lines of mice cannot be attributed to a greater "toxicity" induced by this substance in LS mice.

This presumption is further borne out by experiments using lower AMPT dosage regimens, which are not lethal and produce no apparent behavioral toxicity. When 60 mg/kg, i.p., of AMPT was given every 4 hr for three doses, and brain catecholamines were measured 4 hr after the last dose, a significantly greater depletion of catecholamines was observed in the LS mice. The difference was even more pronounced when a single 125 mg/kg dose of AMPT was given, and brain catecholamine levels were measured 18 hr later. However, neither of these AMPT dosage

regimens produced as great a difference in catecholamine depletion between the LS and SS lines as did a single dose of 250 mg/kg, i.p.

In addition to the possibility of a differential LS/SS susceptibility to AMPT toxicity, there is at least one other indirect effect of AMPT that may confound interpretation of AMPT effects on catecholamine systems. This is the effect of AMPT on body temperature. It had been noted previously that ethanol [6, 32], reserpine,* and adenosine analogues [33] produce different degrees of hypothermia in the LS and SS mice. A difference in hypothermia was also seen following AMPT administration. The degree of hypothermia was greater in the LS mice, and this may be considered a non-specific "toxic" effect of AMPT that could contribute to the inability of LS mice to replenish catecholamine stores as rapidly as SS mice. However, when brain catecholamine levels were measured under conditions of elevated environmental temperature, at which similar degrees of hypothermia were observed in LS and SS mice, a significantly greater catecholamine-depleting effect of AMPT in the LS mice persisted.

These data suggest that there are factors other than differential toxicities or hypothermia that contribute to the greater sensitivity of LS mice than SS mice to AMPT-induced brain catecholamine depletion. Two possibilities are: (1) a difference may exist in the interaction of the competitive inhibitor AMPT with TH or, (2) there may be a difference in the pharmacokinetics of AMPT in the two lines of mice.

The possibility that there is a difference between LS and SS mice in the interaction of TH and AMPT was examined by studying the *in vitro* kinetics of the enzyme. In these studies 6-MePtH$_4$ was used as the reduced-pterin cofactor. The striatum was used as the source of TH in order to have sufficient enzyme activity to perform the kinetic studies, though whole brain was used in all other experiments. The striatum is probably representative of brain TH that is located in dopaminergic neurons. The apparent V_{max} values of striatal TH were similar in LS and SS mice. Tyrosine hydroxylase from SS brains had a slightly higher affinity for the tyrosine substrate than did the LS enzyme. The apparent K_m values for tyrosine of 0.07 mM for SS and 0.11 mM for LS mice are similar to those obtained for TH from whole rat brain (0.14 mM) [34] or sheep caudate nuclei (0.1 mM) [35] using dimethyltetrahydropterin (DMPH$_4$) as cosubstrate. It should be noted that K_m values for tyrosine obtained using either DMPH$_4$ or 6-MePtH$_4$ as the pterin cofactor are higher than tyrosine K_m values obtained when the putative natural cofactor tetrahydrobiopterin is used [36]. Despite the differences in apparent K_m values, the calculated K_i values for AMPT were essentially the same in LS and SS mice. These values of 2.5×10^{-5} M (LS) and 2.7×10^{-5} M (SS) compare to that of 1.7×10^{-5} M for L-α-methyl-p-tyrosine obtained with purified beef adrenal TH [19]. The higher affinity of the SS enzyme for tyrosine and the similar K_i values of AMPT for the enzyme from the two lines of mice indicate that tyrosine should compete for the enzyme slightly more effectively in SS mice than LS mice, in the presence of AMPT. However, it is not likely that the small difference in K_m values would account entirely for the large differences in AMPT effects in the two lines of mice.

Brain AMPT levels in LS and SS mice following a 250 mg/kg dose were found to differ greatly with respect to both peak brain levels attained and the persistence of the substance in the brain. The magnitude and temporal relationship of the difference in pharmacokinetics of AMPT could account for the difference in brain catecholamine synthesis inhibition and brain catecholamine levels observed. Brain levels of AMPT reached a peak at 1 hr in SS mice and began to level off at a low value by 3 hr. In the LS mice, the brain levels of AMPT at 1 hr were 50% higher than in SS mice and were increased further to a slight degree by 3 hr, after which they begin to decline. From 6 to 12 hr after AMPT administration, the brain levels of this amino acid were at least 2-fold higher in LS mice. AMPT levels in LS mice at 12 hr were nearly as high as the peak levels in SS mice at 1 hr. The levels of AMPT in LS and SS mice were most similar at 1 hr, and the declines in catecholamine levels were nearly identical in the two lines of mice for the first 3 hr after AMPT. By 6 hr, the catecholamine levels in SS mice had started to recover at a time when the AMPT levels had fallen off, whereas in LS mice, brain catecholamine levels were still declining and the AMPT levels were still quite high. Widerlov [37] has examined over time the brain concentrations of AMPT following administration of AMPT (250 mg/kg, i.p.) to rats and found them to be similar to the levels we observed in LS mice. Widerlov [37] found peak levels of AMPT at about 4 hr and high levels of AMPT were present even 40 hr after drug administration. The lower brain levels of AMPT in the SS mice apparently account for the less profound catecholamine depletion in these mice than in either LS mice or in rats.

At higher doses of AMPT (500 mg/kg i.p.), the brain levels of AMPT in SS mice were no longer remarkably different from those in LS mice. The peak AMPT brain concentrations at 3 hr were similar in LS and SS mice and only slightly higher than the peak concentration in brains of LS mice at 3 hr after 250 mg/kg of AMPT. The concentration was higher in LS mice at 6 hr, but there was no difference at 12 hr. This 500 mg/kg dose of AMPT, which produced similar brain levels of the substance in the two lines of mice, resulted in similar levels of catecholamine depletion. These data provide further evidence that the differential response to AMPT in LS and SS mice is at least partially explained by pharmacokinetic factors.

The precise reason for the difference in brain AMPT levels between the two lines is not known. One potential site where a pharmacokinetic difference could occur is at the active transport system for passage of neutral amino acids from the plasma into the brain [38, 39]. As a neutral amino acid, AMPT would presumably compete with other neutral amino acids, including the catecholamine precursor, tyrosine, for passage into the brain. It may be that the affinity of the transport system for tyrosine is

* T. A. French and N. Weiner, manuscript in preparation.

greater in SS mice and thus the AMPT is not able to compete for entry into the brain as well as in the LS mice. This could conceivably have a dual effect on catecholamine synthesis in the brain. Not only would there be higher levels of AMPT to interfere with TH activity, there would also be less of the substrate tyrosine available in the brains of LS mice. However, the data on plasma AMPT levels do not support the notion of a difference between LS and SS mice in the affinity of the amino acid transport system for AMPT. The large differences in brain AMPT levels between LS and SS mice are associated with equally large differences in plasma AMPT levels. Thus, the difference in AMPT disposition apparently occurs at, or prior to, entry into the brain and may involve differences in absorption from the peritoneal cavity, first-pass metabolism in the liver, or differences in uptake into peripheral tissues.

It is possible that the observed differences in the apparent K_m values of tyrosine (LS, 0.11 mM; SS, 0.07 mM) for TH in these mice could affect their abilities to synthesize new catecholamines after AMPT administration. Under normal physiological conditions, TH is thought to be saturated with the substrate tyrosine, and the difference in K_m values would not be significant. However, treatment with a competitive inhibitor such as AMPT, which also affects brain tyrosine levels [40], might reduce the tyrosine level and interfere with the interaction of substrate with enzyme to such an extent that the greater affinity of the TH of SS mice for tyrosine would result in a measurable difference in its ability to synthesize catecholamines as compared to the enzyme from LS mice. We did observe that a single treatment with AMPT resulted in a fall in brain tyrosine concentrations from about 0.06 to 0.04 mM 6 hr after administration. It cannot be concluded with certainty whether these changes in brain tyrosine concentrations could affect catecholamine synthesis to a greater extent in LS mice than in SS mice. Nevertheless, the brain tyrosine levels after AMPT treatment are in the range that would be consistent with such an effect.

The present results may also have implications for the difference in sensitivities to ethanol of LS and SS mice. The data suggest there may be differences in the way LS and SS mice handle AMPT, and such differences may suggest that the two lines of mice may also handle tyrosine differently. Recent investigations have shown that a 5 g/kg, i.p., dose of ethanol will decrease rat brain tyrosine levels by over 50% within 10 min [41]. This dose of ethanol is similar to that employed to induce a loss of righting reflex, and the time period required is similar to that needed to detect this loss of righting reflex in LS and SS mice (3.8 to 4.7 g/kg). Ethanol may also differentially affect tyrosine levels in LS and SS mice or may lower tyrosine levels to such an extent that a difference in catecholamine synthesis would occur due to the differences in the affinity of TH for tyrosine (K_m) [42, 43]. Such a mechanism might explain the observed LS/SS differences in catecholamine turnover [13, 17] and may ultimately contribute to the differential LS/SS ethanol sensitivity.

In conclusion, we have found marked differences in the effects of AMPT on brain catecholamine levels in LS and SS mice. The differences may be partially due to hypothermia and/or toxic effects of AMPT, but it most certainly also involves differences in the pharmacokinetics of AMPT and possibly differences in the K_m for tyrosine of TH in the two lines of mice. The determination of the basis for the pharmacokinetic difference awaits further investigation, but the difference probably involves the absorption of the drug from the peritoneal cavity, or first-pass hepatic metabolism.

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