Pharmacology Biochemistry and Behavior, Vol. 66, No. 3, pp. 609–614, 2000

0091-3057/00/$–see front matter

ELSEVIER

PII S0091-3057(00)00263-X

Serotonin Reuptake Is Less Efficient in Taste Aversion Resistant Than in Taste Aversion-Prone Rats

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Received 23 August 1999; Revised 16 January 2000; Accepted 18 January 2000

ELKINS, R. L., T. E. ORR, J. Q. LI, P. A. WALTERS, J. L. WHITFORD, G. F. CARL AND J. L. RAUSCH. *Serotonin reuptake is less efficient in taste aversion resistant than in taste aversion-prone rats.* PHARMACOL BIOCHEM BEHAV **66**(3) 609–614, 2000.—We have previously reported the development of rat lines bred selectively for differences in taste aversion conditionability. Earlier studies demonstrated that the taste aversion resistant (TAR) animals exhibited lower concentrations of brain serotonin and consumed greater amounts of ethanol than their taste aversion prone (TAP) counterparts. In the present study, TAR rats demonstrated significantly less efficient brain serotonin transport compared to TAP rats, but the rat lines demonstrated similar levels of serotonin transporter or V_{max} and similar whole brain paroxetine (a specific serotonin reuptake inhibitor) binding (B_{max}). These results suggest that the rat lines differ in the mechanisms that transport serotonin into nerve endings, but do not differ in the binding of serotonin to the transporter or in the number of serotonin transport sites. The data support the hypothesis that genetically determined differences in the serotonin system contribute to individual differences in taste aversion conditionability. The findings further suggest that differences in serotonin transport may influence the propensity to self-administer ethanol. © 2000 Elsevier Science Inc.

Serotonin Paroxetine Behavioral genetics Serotonin transporter Alcoholism Selectively bred rats Reuptake kinetics Conditioned taste aversion

NUMEROUS species, including rats and humans, will avoid food or drink previously paired with gastrointestinal distress (e.g., nausea). This response, known as a conditioned taste aversion (CTA), is a protective learned rejection of potentially harmful ingestibles. CTAs are remarkably robust, and are frequently acquired after only a single conditioning trial (23–25,54). Flavor novelty facilitates CTA acquisition (8), but even highly familiar flavors can become aversive following multiple conditioning trials (9).

Among humans, CTAs are both prevalent and long lasting (5,22,32,33). In a survey of nausea based CTAs, 65% of 517 humans reported one or more such extant aversions (33). Some of these consummatory aversions involved smell, textures, or appearance. However, taste was involved in 83% of the aversions, and taste was by far that aspect of an ingestible most likely to be rendered aversive. Alcohol aversions following episodes of alcohol-induced nausea are among the most commonly reported TAs in humans (5,33,52). Nonetheless, variations in propensities for humans to acquire CTAs are prevalent, and failures to condition are common within TA-based treatments of substance abuse (13).

Rats, like humans, display marked individual differences in TA conditionability (11). Moreover, the rat is a recognized animal model for human consummatory aversion conditionability and aversion therapy treatment (33). Selectively bred lines of TA-prone (TAP) and TA-resistant (TAR) lines were developed to serve as an animal model for individual differences in consummatory aversion conditionability. Bidirectional line selection was undertaken using a saccharin-flavored solution as the conditioned stimulus and the postingestional consequences of an injection of cyclophosphamide, an agent that produces nausea and emesis in humans, as the unconditioned stimulus. Selection was continued for 28 generations and produced two lines of rats that differ markedly in TA

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conditionability across a wide range of conditioned and unconditioned stimuli (12,14–16). The marked TAP and TAR line divergence has confirmed a robust genetic control of individual variability in TA conditionability. The gradual nature of the divergence is consistent with polygenetic control of TA conditioning propensities (47).

Although the TAP and TAR lines were developed as an animal model of TA-based treatment of human substance dependence, their value extends beyond the study of such aversion therapy treatments. TAR rats, having had no prior experience with ethanol, consumed considerably more ethanol than TAP rats in a two-bottle, free-choice paradigm (46). This finding suggests that the selectively bred line differences in CTA acquisition modulate line differences in ethanol consumption. Consequently, we have proposed that, within humans, the propensity to acquire CTAs may afford aversion-prone individuals some protection from alcohol dependency formation by limiting their ingestion of alcoholic beverages (46). This hypothesis is similar to that which holds that low harm avoidance per se is linked to an increased propensity for the initiation and continuation of alcohol drinking (3).

Initial comparisons of neurotransmitter levels in these rat lines revealed that brains of TAR rats have lower levels of serotonin and higher levels of norepinephrine than do the brains of TAP rats (45). Both of these monoamines have been identified as putative neurotransmitter substrates of TA conditioning (1,19,34–36,57), and have been implicated in the control of ethanol self-administration (55). In addition, serotonin function has been related to the general phenomenon of harm avoidance (3,30,49), and harm avoidance, in turn, has been associated to different patterns of alcohol preference (3,21). Serotonin levels in the synapse are regulated through the action of the serotonin transporter that actively removes serotonin from the extracellular space, thereby constituting the "off" signal in the synapse. Serotonin transport differences have been specifically associated with differences in ethanol ingestion in several studies (25–27,39,40,43,51). Thus, as an extension of the earlier findings of lower serotonin levels in TAR rats, it was hypothesized that TAP and TAR rats would differ in serotonin transport mechanisms. In the present study, these rat lines were investigated for differences in brain synaptosomal serotonin transport and for differences in serotonin binding sites in brain cell membrane preparations, as measured by paroxetine binding.

METHOD

Materials

[Phenyl-6′-^{3}H]-paroxetine (16.4 Ci/mmol) and 5-[1,2-^{3}H(N)]-serotonin (23–30 Ci/mmol) were purchased from DuPont/NEN, Boston, MA. Whatman GF/F 25 mm (dia) glass microfiber filters were obtained from Fisher Scientific, Atlanta, GA. Cellulose nitrate [0.45 μ, 25 mm (dia)] filters were provided by Millipore Corp., Marlborough, MA. Unlabeled serotonin was obtained from Fluka Chemicals, Ronkonkoma, NY, and unlabeled paroxetine was obtained from Smith, Kline and Beecham, King of Prussia, PA. All other chemicals were purchased from Sigma Chemical Co., St Louis, MO.

Animals

Twelve TAP rats and 12 TAR rats of the 28th generation of selective breeding were chosen for isolation of synaptosomes for determination of serotonin uptake. The rats ranged in age from 7 to 8 months. Pairs of TAP and TAR rats, matched for weight and sex, were killed by decapitation at the same time. Each brain was excised, the cerebellum dissected away, the remaining cortex and midbrain weighed, and synaptosomes prepared as described below. Assays were performed on the synaptosomes from a matched pair of rats. A TAP and a TAR were paired for sacrifice to ensure that small variations in the procedure from one assay to the next would not bias the results, and because it was not possible to include all samples in a single assay.

Five male TAP rats and five male TAR rats of the 32nd generation (as stated above, selection was terminated after 28 generations) were chosen for isolation of membranes for determination of paroxetine binding. The rats ranged in age from 4 to 7 months. A TAP and a TAR rat were matched for weight and killed by decapitation at the same time so that isolation of membranes and binding studies were paired. Each brain was excised, the cerebellum dissected away, the remaining cortex and midbrain weighed and membranes prepared.

Preparation of Rat Brain Synaptosomes

Crude rat brain synaptosomes were prepared by a modification of a method described elsewhere (38). The midbrain and cortex were homogenized in 0.32 mol/l sucrose containing 10 μmol/l iproniazid (10 ml/g) using a Dounce homogenizer (four to six strokes). The homogenate was centrifuged at 2000 × *g* for 10 min, the pellet discarded, and the supernatant centrifuged again under identical conditions. The second pellet was discarded, and the supernatant was centrifuged at 10,000 × *g* for 20 min. The resulting pellet, containing synaptosomal material, was resuspended in 0.32 mol/l sucrose at approximately 2 ml/g of original starting material. This suspension was used for the assays. Protein concentrations were determined by the method of Lowry et al. (37) using bovine serum albumin as standard.

Serotonin Uptake in Rat Brain Synaptosomes

Serotonin uptake was assayed by a modification of the procedure described by Rausch et al. (50). To 900 μl modified Krebs buffer (NaCl, 131.7 mmol/l; $MgSO_4$, 3.3; Tris, 5.0; KCl, 4.3; $CaCl_2$, 2.5; Dextrose, 5.5; pH 7.4 at 37°C) was added 0.625 (lowest serotonin concentration) or 1.25 (four higher serotonin concentrations) μCi [^{3}H]-serotonin. Nonradioactive serotonin was added to yield concentrations of 0.025, 0.05, 0.1, 0.5, and 1.0×10^{-6} mol/l. Incubation was started by adding 100 μl of the synaptosomal suspension (6 mg protein/ml) and continued in duplicate for 5 min in a water bath at 37°C. The uptake was terminated by filtration through cellulose nitrate filters (0.45 μm), and washed twice with 2 and 5 ml of cold (4°C) buffer. A blank was generated for each set of duplicates by adding 1×10^{-4} mol/l paroxetine to an identical tube before addition of the synaptosomes. Radioactivity collected on the filter was assayed by liquid scintillation spectrometry. Active uptake was determined by subtracting blank values (paroxetine added) from total uptake (activity retained on the filter). Kinetic constants (K_m and V_{max}) were then calculated according to the methods of Cleland (2).

Preparation of Rat Brain Membranes

Rat brain membranes were prepared by a modification of the method described by Lawrence et al. (29). Brain tissue was homogenized in ice-cold 0.25 mol/l sucrose (1:15 w/v) using a Kinematic Polytron (Brinkman Instruments, Westbury, NY) on setting 6 for 10 s. The homogenate was diluted 1:1

with cold 0.25 mol/l sucrose and centrifuged at 1,000 × *g*, 12 min, 4°C. Two-thirds of the supernatant (~20 ml) was diluted 1:2 with Tris buffer (~40 ml) (50 mmol/l TrisHCl, 120 mmol/l NaCl, 5 mmol/l KCl, pH 7.4) and centrifuged at 28,000 × *g*, 10 min. The final pellet was resuspended in Tris buffer at 1 mg protein/ml and used in the paroxetine binding assay.

Paroxetine Binding in Rat Brain Membranes

To 900 μl of Tris buffer (see above) containing ^{3}H-paroxetine (16.4 Ci/mmol) at concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, and 1.0 × 10^{-9} mol/l was added 100 μl of rat brain membrane preparation (1 mg protein/ml). Samples were incubated in duplicate in a shaking water bath for 1 h at room temperature. Blanks were generated by adding unlabeled paroxetine to a concentration of 10^{-4} mol/l. Incubations were terminated by filtration of each sample through Whatman GF/F glass microfiber filters, presoaked in 0.3% polyethylenimine for 1 h. The radioactivity retained on the filter was assayed by liquid scintillation spectrometry. Specific binding was determined by subtracting blank values from total binding.

RESULTS

Serotonin Uptake Into Brain Synaptosomes

The velocities of the uptake of serotonin into the synaptosomes were calculated for each of the serotonin concentrations in each of the brains. Lineweaver-Burk plots of these data were constructed using linear regression analysis. These plots revealed a difference in the K_m between TAP and TAR rats (abscissal intercept = $-1/K_m$, Fig. 1), but no difference in maximal velocity (V_{max}) between the lines (ordinal intercept = $1/V_{max}$, Fig 1).

To enable statistical analysis, the K_m and the V_{max} for serotonin uptake were calculated separately for each of the 24 animals. Table 1 compares the average K_m and the average V_{max} for the TAP rats and the TAR rats. Statistical comparison of the average K_m and the average V_{max} between TAP rats and TAR rats validated the conclusion from the plotted composite data that the two lines of rats had very similar maximal uptake velocities (V_{max}, Table 1), but the rat lines differed significantly in their response to varying concentrations of serotonin in the incubation medium (K_m, Table 1).

TABLE 1

COMPARISON OF KINETIC CONSTANTS OF SEROTONIN UPTAKE INTO SYNAPTOSOMES ISOLATED FROM BRAINS OF TAP AND TAR RATS

	K_m 10^{-8} mol/l*	V_{max} pmol/mg/min*
TAP	11.13 ± 1.59	7.03 ± 0.77
P	<0.005	>0.1
TAR	14.21 ± 3.08	7.27 ± 1.14

*The values represent the means of 12 independent determinations ± standard deviation.

Paroxetine Binding to the Serotonin Transporter in Brain Membranes

The binding of paroxetine, a potent and specific serotonin reuptake blocker (4), to membrane preparations was calculated as a function of paroxetine concentration. Scatchard plots were constructed by linear regression analysis of the data from each line of rats. The two plots were nearly coincident (Fig. 2), indicating that there were no differences in either the dissociation kinetics of paroxetine from membranes or in the maximum binding of paroxetine to membranes between TAP and TAR rats.

In addition to the composite Scatchard analysis, the mean K_d and the mean B_{max} values were calculated separately for

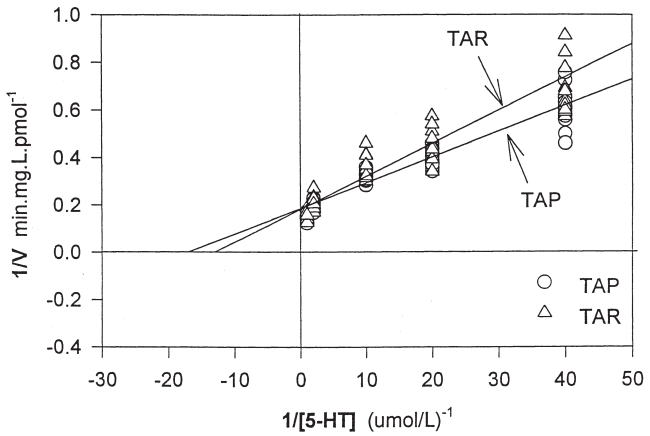

FIG. 1. Lineweaver-Burk plots of the reciprocal of the serotonin concentration vs the reciprocal of the velocity of the uptake of serotonin into synaptosomes isolated from the brains of TAP and TAR rats. The uptake velocities of labeled serotonin into synaptosomes was determenied as described in the Method section. Points were plotted at five different concentrations for each of 12 TAP rats and each of 12 TAR rats. The best fit curves were generated by linear regression. Comparison of the ordinal intercepts indicates no difference in V_{max}s between TAP and TAR rats. Comparison of the abscissal intercepts indicates a difference in K_ms between TAP and TAR rats (see Table 1).

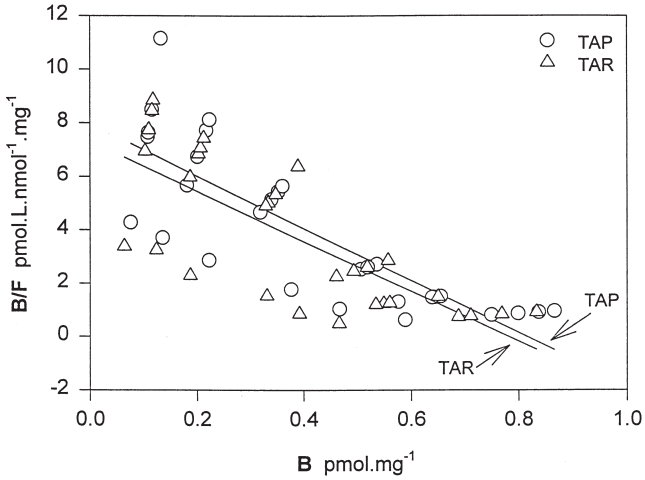

FIG. 2. Scatchard plots of the ratio of bound/free paroxetine vs. bound paroxetine in plasma membranes isolated from the brains of TAP and TAR rats. Membranes were isolated by differential centrifugation as described in the Method section. Points were plotted for six different concentrations of paroxetine for each of five different membrane preparations from the brains of five TAP and five TAR rats. The best fit curves were generated by linear regression. The similarities of the abscissal intercepts and the slopes of the plots indicate that there is little difference between TAP and TAR in either the B_{max}s or the K_ds of the isolated membranes for paroxetine.

each line (TAP and TAR). There was no apparent difference between TAP and TAR rats in the affinity with which paroxetine was bound (K_d, Table 2). Nor was there a significant difference between TAP and TAR rats in the concentration of transport sites on the membranes (B_{max}, Table 2). That there was no between-line difference in B_{max} for paroxetine is consistent with the absence of a between-line difference in V_{max} for serotonin uptake.

DISCUSSION

The data presented in this report indicate that there is a difference between the serotonin transporters of the TAP and the TAR rats, suggesting that serotonin transport is involved in the taste aversion conditioning by which the lines were defined. The lack of a difference between the lines in the binding of paroxetine, a competitive inhibitor of serotonin transport (4), to plasma membranes (K_d) suggests that a difference exists in the serotonin transporter protein that affects transport but not binding. The observations that the two lines of rats exhibit a similar V_{max} for serotonin transport and a similar B_{max} of paroxetine binding indicate there is no difference in the number of serotonin transport sites between the two lines.

Paroxetine binding and serotonin transport are fundamentally different measures insofar as paroxetine binds to the transporter protein with a fixed stoichiometry short of any transport process. The measurement of the K_m of serotonin transport is alternatively a measure of the affinity of the protein for the net transport of serotonin across the cell membrane. One interpretation of these results is that the transport process for serotonin has three components—an initial binding of serotonin to the transport protein, movement of serotonin through the cell membrane, and release of serotonin from the transporter inside the cell. If the affinity of paroxetine for the transporter is reflective of the affinity of serotonin for its initial binding to the transporter site, then an interpretation of these data could be that the serotonin transport differences between lines is not related to that first phase of serotonin's initial attachment to the transporter. In any case, the data clearly indicate a difference in the transport affinity between lines. Although it is possible that other factors involved in the actual movement of the serotonin through the membrane may account for this difference, the most obvious possibility is the transporter protein itself. The affinity difference could be caused by a between-line difference in the structure of the protein, or in some post-translational modification.

Phosphorylation of the transporter is one such posttranslational mechanism. It could alter the kinetic properties of the transporter, playing a role in its affinity for its substrate (49). These processes in the rat may be relevant to the human case, because the rat serotonin transporter gene is 92% homologous to the human serotonin transporter gene (48). At the same time, other genes influencing the structure or posttranslational modification of the serotonin transporter could be operative in expression of the identified phenotypic differences in transport affinity noted here.

The greater efficiency of the serotonin reuptake transporter of the TAP rats compared to TARs suggests that TAP rats clear serotonin from the synaptic cleft more quickly than the TARs. Orr et al. (45) showed that the TAP line had higher total concentrations of serotonin in the brain than did the TAR rats. If the serotonin in the TAR line remains in the extracellular space longer or at higher concentrations, the neurotransmitter is being exposed to degradation at a greater rate than the serotonin in the TAP line because it is more likely to diffuse or be transported into adjoining glial cells where it would be exposed to catabolism. Consequently, the total serotonin concentration in the TAR brain would be expected to be more depleted than serotonin in the TAP brain.

With the exception of the TAP and TAR lines, all other selectively bred rat lines that differ in ethanol acceptance were derived through selective breeding based on ethanol intake. The TAP and TAR lines were selectively bred for efficient or inefficient TA conditionability based on administration of cyclophosphamide, an emetic agent having no psychotrophic properties, after exposure of the animal to the novel taste of saccharin. Although the lines were developed based strictly on conditionability differences, they exhibit significant differences in alcohol preference as well (46). Therefore, the data presented here are consistent with the hypotheses that 1) the selectively bred differences in the serotonin transporter system between TAPs and TARs contribute to between-line differences in both TA conditionability and ethanol preference, and that 2) the phenotypic expression of taste aversion and alcohol preference share common genetic origins related in part to serotonin transporter function. However, as previuosly noted, the gradual response to selection of TA conditionability (16) is consistent with polygenetic control. The serotonin transporter hypotheses have heuristic and potential explanatory value, but additional aspects of the serotonin system or the norepinephrine system (45) plus other as-yet unidentified processes may contribute to the TAP and TAR differences in TA conditionability and ethanol preferences.

Early support for the hypothesis that TA conditionability modulates ethanol self-administration was provided by a study in which the postingestional consequences of ethanol intake of Sprague–Dawley rats were circumvented through the use of a surgically developed intragastric fistula (6). Most of the rats whose stomachs were pumped to promptly remove a 10% ethanol solution ingested as a novel substance on day one increased their alcohol intake on day 2; all fistula-equipped control subjects whose stomachs were not pumped to remove ingested alcohol on day 1 displayed greatly diminished alcohol intake on day 2. The taste of alcohol caused no more than a transitory neophobia in the experimental rats who, due to alcohol removal, had experienced diminished postingestional consequences of alcohol intake. The authors (6) concluded that the persistent reticence of many randomly bred rats to ingest alcoholic solutions may not have been due to any strong or long standing gustatory/olfactory rejection, but instead may have arisen from the rapid acquisition of a CTA to ethanol based on negative postingestional consequences of ethanol intake. However, there is a wide range of variability with respect to susceptibility to acquisition of CTAs. An identification of

TABLE 2

COMPARISON OF PAROXETINE BINDING IN PLASMA MEMBRANE PREPARATIONS FROM BRAINS OF TAP AND TAR RATS

	K_d 10^{-8} mol/l*	B_{max} pmol/mg/prot*
TAP	0.0932 ± 0.0287	0.7584 ± 0.0837
TAR	0.0876 ± 0.0240	0.6914 ± 0.1130

*The values represent the means of five independent determinations ± standard deviation. There were no significant differences in paroxetine binding between the membranes isolated from the brains of TAP and TAR rats.

the neurotransmitter receptor–effector systems that predispose some randomly bred rats toward efficiency with respect to consummatory aversion learning may be important for understanding how different initial conditions may result in different behavioral outcomes to the same initial access to ethanol. The data presented here support the involvement of serotonin transporters in these processes.

The likelihood that selectively bred differences in the serotonin system of TAP and TAR rats will prove to be an important substrate of alcoholism vulnerability is enhanced by numerous reports of other investigators. For example, preclinical and clinical reports of decreased ethanol intake in conjunction with an increased tone of serotonin central processes are summarized in a recent review (55). Consistent with these reports, it has been repeatedly shown that treatment with serotonin reuptake inhibitors reduces voluntary ethanol drinking in rats (25–27,39,40,51) and humans (55). This finding has been observed in the P (alcohol-preferring) rat that was selectively bred for the behavioral phenotype of high alcohol oral self-administration (41,51). Moreover, P rats have lower concentrations of serotonin in forebrain and limbic regions that their NP (nonalcohol-preferring) counterparts (26). Nevo and Harmon (44) conclude that one consistent marker across lines of alcohol drinking rats would appear to be low levels of serotonin in several brain areas. Myers et al. (42) concur and argue that if low serotonin concentrations are in fact related to increased alcohol consumption, then rats bred for a behavioral marker other than alcohol self-administration but having low concentrations of central serotonin should also be high drinkers of alcohol. The TAR rats, which have a low concentration in brain serotonin concomitant with high ethanol acceptance (45,46), are cited by Myers et al. (42) as fulfilling this expectation. The present indications of a difference between the 5-HT transporters of TAP and TAR rats advances an experimental focus that may clarify possible linkages of serotonergic involvement in TA conditionability and ethanol self-administration. There are no data yet available on the effect of serotonin reuptake inhibitors on the ethanol self-administration in the TAR line.

Naturally occurring TA conditioning processes may afford aversion prone individuals some protection from dependency formation by limiting their ingestion of alcoholic beverages (46). Moreover, conditioned taste aversions are sometimes used as treatments for both alcohol (13,18,56) and cocaine dependence (13,17,20). Some evidence suggests that successful conditioning may reduce conditioned craving and the risk of relapse (13). However, like alcoholics and randomly bred rats, some cocaine-dependent recipients fail to develop conditioned aversions to the target substances during emetic treatments (17). Many alcohol or cocaine-dependent recipients of nausea-based aversion therapies fail to acquire CTAs, despite having nausea reactions that are indistinguishable from those of successfully conditioned subjects (10,17). These observations indicate that many people may be resistant to CTA acquisition. Lacking such natural CTA protection, TA resistant individuals may be at high risk for developing dependencies on alcohol and other self-administered psychoactive substances.

Several studies suggest a relationship between harm avoidance and serotonin transporter function (30,49). Serotonin transport has also been associated with differences in ethanol ingestion leading to the suggestion that differences in alcohol preference may be mediated by the same mechanisms responsible for harm avoidance (21,30). However, TAP and TAR rats do not differ from each other in their general patterns of harm avoidance. Instead, they have been selectively bred specifically for their propensity to avoid an aversively conditioned taste stimulus. No significant between-line learning differences have been found with respect to shuttle box avoidance, food-reinforced operant bar-press responding, or radial arm maze food procurement (28). TAP and TAR rats exhibit pronounced but highly circumscribed learning ability differences that appear only within the described gustatory aversions. Indeed, it was on the taste aversion parameter alone upon which they were selectively bred.

In summary, rat lines selectively bred for differences in taste aversion conditioning also exhibit differences in alcohol preference and in the ability to transport serotonin into nerve endings. Although the correlative nature of these results does not permit causal conclusions, it is suggested that these results could be explained by assuming that genetic differences result in differential expression of the serotonin transporter, which in turn, influence both taste aversion conditioning and alcohol acceptance. Much research is now underway to identify specific alleles that may influence vulnerability to alcoholism or to behaviors related to alcoholism (7,31,53). The presently reported findings support the hypotheses that the serotonin transporter gene is a gene involved in alcoholism vulnerability, and that there is a relationship of CTAs to that vulnerability and to potential treatments of consummatory patholologies. It is hypothesized that molecular and functional characterizations of the TAP and TAR serotonin transporters will reveal genetic polymorphisms that are potentially related to TA conditionability and alcohol self-administration in the TAP and TAR lines. Alternatively, some posttranslational modifications of the serotonin transporter protein may be found to influence the different TAP and TAR behavioral phenotypic responses to TA conditionability and ethanol self-administration.

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