**Interaction Between Serotonin Transporter Gene Variation and Rearing Condition in Alcohol Preference and Consumption in Female Primates**

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**Background:** Serotonin neurotransmission and limbic-hypothalamic-pituitary-adrenal (LHPA) axis hormones are thought to be involved in the reinforcement of alcohol intake and contribute to the risk for alcoholism. In humans and macaques, a promoter polymorphism that decreases transcription of the serotonin transporter gene is associated with anxiety and altered LHPA-axis responses to stress, and in female macaques, exposure to early-life stress alters LHPA-axis activation in response to alcohol. We wanted to determine whether serotonin transporter gene promoter variation (rh-5HTTLPR) and rearing condition would interact to influence alcohol preference in female rhesus macaques. Because of the involvement of stress and LHPA-axis activity in withdrawal symptoms, we also wanted to determine whether serotonin transporter gene variation and rearing condition would influence changes in the patterns of alcohol consumption across a 4-week alcohol consumption paradigm.

**Methods:** Female macaques were reared with their mothers in social groups (n=18) or in peer-only groups (n=14). As young adults, they were given access to an aspartame-sweetened 8.4% alcohol solution and vehicle for 1 hour per day, and volumes of consumption of alcoholic and nonalcoholic solutions were recorded. Serotonin transporter genotype (l/l and l/s) was determined using polymerase chain reaction followed by gel electrophoresis.

**Results:** We found interactions between rearing condition and serotonin transporter genotype, such that l/s peer-reared females demonstrated higher levels of ethanol preference. We also found an effect of rearing condition on the percentage change in alcohol consumed during the 6 weeks as well as a phase by rearing interaction, such that peer-reared animals progressively increased their levels of consumption across the course of the study. This was especially evident for peer-reared females with the l/s rh-5HTTLPR genotype.

**Conclusion:** These data suggest a potential interaction between serotonin transporter gene variation and early experience in vulnerability to alcoholism.

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**Alcoholism is a Relapsing, lifetime illness that is notoriously difficult to treat.** Although it is a complex disorder, with multiple subtypes and clinical pictures, one defining feature of alcoholism is the compulsive use of ethanol, often in the face of negative social and psychological consequences. Both positive and negative reinforcement are thought to be critically involved in the transition from casual alcohol use to compulsive alcohol-seeking behavior. While the positive reinforcing effects of alcohol are essential to the initiation and early maintenance of intake, recent studies suggest that alcohol-seeking behavior related to alleviation of symptoms during abstinence (negative reinforcement) is equally, if not more, effective in maintaining alcohol use. Therefore, when considering risk factors for alcohol dependence, it is important to consider not only systems that are involved in alcohol reward but those activated following alcohol exposure, during acute withdrawal and abstinence.

Among the systems involved both in positive reinforcement of alcohol-induced reward and negative reinforcement of alcohol withdrawal is the limbic-hypothalamic-pituitary-adrenal (LHPA) axis. Acutely, exposure to ethanol increases hypothalamic release of corticotropin-releasing hormone (CRH) and arginine vasopressin. This is followed by an increase in pro-opiomelanocortin synthesis in both the adenohypophysis and the intermediate lobe of the pituitary gland. Posttranslational cleavage of the pro-opiomelanocortin precursor produces multiple peptides, including β-endorphin (β-EP) and corticotropin, the former peptide producing some of the rewarding and re-
inforcing effects of alcohol.\textsuperscript{6,7} Corticotropin is released into the peripheral circulation to stimulate the synthesis and release of glucocorticoids, which are known to potentiate the positive reinforcing effects of drugs of abuse, from the adrenal cortex. Increases in CRH release also occur in forebrain structures during acute exposures to alcohol and for up to 12 hours of withdrawal,\textsuperscript{8} and release of CRH in the central nucleus of the amygdala is thought to contribute to anxiety associated with acute alcohol withdrawal.\textsuperscript{9,10}

The serotonin system is also involved both in reinforcement of alcohol intake and symptoms of withdrawal, and a number of studies have shown that the serotonin system modulates CRH release and that there are reciprocal interacting influences between the LH/axis and central serotonin activity.\textsuperscript{11,12} While serotonin release following consumption of alcohol is involved in activation of reward pathways, neuroadaptive diminutions in release following alcohol exposure can lead to pain, dysphoria, and depression.\textsuperscript{13} Much focus in the area of alcohol research has been on the serotonin system. Serotonin is one of the key neurotransmitters to be released in response to alcohol.\textsuperscript{14} In rodents, alcohol preference is associated with decreased levels of serotonin and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in relevant areas of the brain as well as a decrease in the number of serotonergic neurons in the raphe nuclei.\textsuperscript{15} Among alcohol-prefering rats, this serotonin deficit is maintained even following administration of ethanol.\textsuperscript{16} In humans, low cerebrospinal fluid levels of 5-HIAA are associated with alcohol-seeking behavior and alcoholism.\textsuperscript{17,18} This has been observed among nonhuman primates as well.\textsuperscript{19-22}

Variations in many of the genes that encode receptors, enzymes, and transporters for factors released in response to alcohol have also been studied in association with alcoholism. A common insertion-deletion polymorphism in the promoter region for the serotonin transporter gene promoter variation have been associated with alcohol-seeking behavior and traits that relate to certain subtypes of alcoholism.\textsuperscript{27-29} Orthologous to a number of studies have shown that the serotonin system–related genes, maternal deprivation, and stress in the etiology of alcohol consumption. What follows is a study focusing on female drinking patterns in adolescent macaques given simultaneous access to alcoholic and nonalcoholic aspartame-sweetened solutions for 1 hour a day for a period of 6 weeks. Both preference for alcohol and changes in patterns of alcohol consumption with repeated exposures were determined. Since serotonin, β-EP, and corticosteroids are involved in the reinforcement of alcohol intake and because peer-rearing and serotonin transporter gene promoter variation have been associated with alcohol consumption in human and animal populations, we predicted that rh5-HTTLPR genotype and rearing experience would interact to influence preference for alcohol in female rhesus macaques. As noted, the serotonin system and hypothalamic-pituitary-adrenal axis interact centrally. Because PR females have been shown to have higher levels of LH/axis activation following administration of alcohol and since dysregulated release of serotonin and CRH are related to symptoms of anxiety and stress, we also wanted to determine the effects of these variables on the maintenance of voluntary alcohol intake across the course of a 6-week alcohol consumption paradigm.

### METHODS

#### ANIMALS

Animals were either PR or mother-reared (MR). The former condition deprives animals of parental input and the opportunity to learn appropriate social behaviors and context during early development and is a model for early-life stress, whereas the latter is meant to approximate natural conditions. These rearing conditions have been described in detail elsewhere.\textsuperscript{21,27,30} Briefly, MR animals (n=18) were reared for the first 6 months of life with their mothers and fathers in social groups comprising 8 to 12 adult females (about half of whom had same-aged infants) and 2 adult males. Peer-reared animals (n=14) were separated from their mothers at birth and hand-reared in a neonatal nursery for the first 37 days of life. For the first 14 days, they were housed in an incubator and hand-fed. From day 14 until day 37, they were placed alone in a nursery cage and provided a blanket and a terry cloth–covered, rocking surrogate. A bottle from which the infants would feed was fixed to the surrogate. At 37 days of age, they were placed in a cage with 3
Figure 1. Effects of rh5-HTTLPR genotype (l/l or l/s) and rearing condition (mother reared [MR] or peer reared [PR]) on alcohol preference in female rhesus macaques. Values are expressed as mean ± SEM for the percentage of alcohol consumed.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MR</th>
<th>PR</th>
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<tbody>
<tr>
<td>l/l</td>
<td>30%</td>
<td>40%</td>
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<tr>
<td>l/s</td>
<td>20%</td>
<td>10%</td>
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There was a significant main effect of serotonin transporter gene variation and early-life stress on maintenance of alcohol consumption across the course of the drinking paradigm, weekly means of alcohol consumed were determined, and the influences of rh3-HTTLPR genotype and rearing condition on weekly averages of alcohol consumption in grams per kilogram were analyzed using repeated-measures analysis of variance. There were 14 MR l/l, 4 PR l/l, 10 PR l/s, and 4 PR l/s females in the study. Since animals with the x/l and s/s genotype were rare, they were excluded from all analyses. Alcohol consumption rates were available for 6 animals for only 4 or 5 weeks, and vehicle consumption rates were unavailable for 1 animal. All analyses were performed using StatView Statistical software (SAS Institute, Cary, NC). Criterion for significance was set at P < .05.

There was a significant main effect of serotonin transporter gene variation on alcohol preference (Figure 1) (F(1,13) = 5; P < .04). Alcohol preference for female macaques with the l/s genotype (mean ± SEM alcohol preference was 60% ± 10% of the animals were MR and 44% ± 10% were PR. All animals lived in their respective social groups throughout the study and received identical treatment. Protocols for the use of experimental animals were approved by the institutional animal care and use committee of the National Institute of Alcoholism and Alcohol Abuse, Poolesville, Md.

**ALCOHOL CONSUMPTION**

Study animals were 32 young adolescent, female rhesus macaques, obtained from 4 birth-year cohorts, ranging in age from 3.3 to 3.7 years (mean age, 3.4 years) at the initiation of the alcohol self-administration study. Animals were allowed to freely consume an aspartame-sweetened 8.4% (volume-to-volume ratio) alcohol solution for 1 hour a day, 4 days a week for 2 weeks.20,21,37 Briefly, this standardized method consisted of 3 phases: (1) Spout training. The animals were trained for an hour a day across a 1-week period to drink from nipple-like spouts that dispensed aspartame-sweetened water. This phase lasted 5 days, at which point all animals consumed more than 30 mL of the vehicle. (2) Initial alcohol exposure. This phase was designed to assure that all animals experienced the pharmacological effects of alcohol before beginning the experimental phase of the study. To begin this phase, the color of the sweetened vehicle was changed, and alcohol was added to the vehicle to produce an 8.4% volume-volume ratio alcohol solution. During the initial alcohol exposure phase, animals were given free access to the alcohol solution for an hour each day. Each of the animals included in this phase of the study fulfilled a preestablished criterion of consuming more than 0.67 g/kg body weight of the alcohol solution on 2 or more occasions. Once all animals met the criterion, a second bottle containing sweetened vehicle was added. Thereafter, both the nonalcoholic and 8.4% alcoholic sweetened solutions were available in addition to normal drinking water for an hour each day. No special methods, such as deprivation of food or water, were used to induce drinking, and animals established stable consumption patterns within 2 weeks. (3) Experimental period. During the 6-week experimental phase, alcohol and vehicle were dispensed 4 days a week (Monday–Thursday) from 1300 to 1400 while the animals were in their home-cage environment. Animals' weights ranged from 4.2 to 9.3 kg (mean ± SD weight, 5.8 ± 1.2 kg).

**GENOTYPING**

Using standard extraction methods, DNA was isolated from whole blood and collected from the femoral vein after the animals had been given ketamine anesthesia (15 mg/kg, intramuscular). The serotonin transporter gene promoter region (rh5-HTTLPR) was amplified from 25 ng of genomic DNA with flanking oligonucleotide primers (stpr5, 5'-GGGCTTGCCGCTCAGAATGC; 31, 5'-CAGGGGAGATCTCCTGGGAGG) in 15-μL reactions using Platinum Taq and the PCRX Enhancer System kit (Invitrogen, Carlsbad, Calif), according to the manufacturer's protocol. Amplifications were performed on a thermocycler (9700) (Perkin-Elmer, Fremont, Calif) with 1 cycle at 96°C for 5 minutes followed by 30 cycles of 94°C for 15 seconds, 60°C for 15 seconds, 72°C for 30 seconds, and a final 3-minute extension at 72°C. Amplifications were separated by electrophoresis on a 10% polyacrylamide gel, and the short (s) (398-bp) and long (l) (419-bp) alleles of the rh5-HTTLPR were identified by direct visualization following ethidium-bromide staining.

**DATA ANALYSIS**

To assess the effects of rh5-HTTLPR genotype and rearing condition on alcohol preference, 2-way analysis of variance was conducted. Animals were assigned nominal independent variables according to rearing condition (MR or PR) and rh5-HTTLPR genotype (l/l or l/s), and the influence of these variables on alcohol preference was determined. Average volumes (mL) of alcoholic and nonalcoholic solutions consumed during the course of the drinking study were calculated, and alcohol preference was determined by dividing the volume of 8.4% alcohol solution consumed by the total volume of solution consumed and multiplying by 100 (% milliliters of alcoholic solution/milliliters of alcohols solution + milliliters of nonalcoholic solution) × 100).

To determine the influence of serotonin transporter gene variation and early-life stress on maintenance of alcohol consumption across the course of the drinking paradigm, weekly means of alcohol consumed were determined, and the influences of rh5-HTTLPR genotype and rearing condition on weekly averages of alcohol consumption in grams per kilogram were analyzed using repeated-measures analysis of variance. There were 14 MR l/l, 4 PR l/l, 10 PR l/s, and 4 PR l/s females in the study. Since animals with the x/l and s/s genotype were rare, they were excluded from all analyses. Alcohol consumption rates were available for 6 animals for only 4 or 5 weeks, and vehicle consumption rates were unavailable for 1 animal. All analyses were performed using StatView Statistical software (SAS Institute, Cary, NC). Criterion for significance was set at P < .05.
more than MR animals across the course of the 6-week al-
terior to the development of psychopathology and neu-
ropsychiatric disease as well.41,42 Today, it is widely accepted
that neuropsychiatric disorders are complex traits, driven
by both environmental and genetic influence, and that,
moreover, there are potential gene–gene and gene × envi-
ronment interactions that may be at the root of the devel-
opment of some personality traits and neuropsychiatric dis-
eases.43,44 With the advent of modern molecular and
statistical tools, we are now capable of refining our ap-
proach so that specific gene × environment interactions in
the development of psychiatric disease can be revealed.

Alcohol abuse and alcoholism are among the psychi-
atric disorders thought to be influenced strongly by both
genetic and environmental factors. Individuals who ex-
perience early-life psychosocial stressors, such as abuse or
loss of a parent, are at increased risk for anxiety and de-
pression, known risk factors for alcoholism, and parental
monitoring is known to modulate the risk for alcohol abuse
in adolescents.45 The 5-HTTLPR s allele has been demon-
strated to be associated with anxiety, neuroticism, and as-
associated traits in numerous studies,43 and recent studies
have demonstrated carriers of the s allele to be more sus-
ceptible to major depression in the face of repeated stress.39

The obvious strength of animal studies is that environ-
mental factors can be controlled, such that relative con-
tributions or potential interactions between genes and en-
vironment can be examined.46 Moreover, the animal model
allows for us to study mechanisms of these interactions,
since molecular and environmental interactions can be
observed as they relate to behaviors that are potential
contributors to the susceptibility, pathogenesis, and pro-
gression of psychiatric illnesses.

Some studies have shown an association of the l/s sero-
tonin transporter genotype with alcoholism.28 However,
other studies have produced negative results.37 These
contradictory results could be attributable, in part, to vari-
ations in early experience or in sex composition among
different populations of study. In the present study, we
report that there is an association between serotonin trans-
porter gene promoter variation and ethanol preference
in female rhesus macaques. The effect of serotonin trans-
porter genotype, however, is environmentally depen-

dent. Perhaps the most interesting aspect of this study is that a genotype thought to confer risk for a wide variety of psychopathological illnesses and traits exerts its effect on alcohol preference and consumption only when animals are reared in an environment that models parental absence or neglect. This interaction is observed only in relation to alcohol consumption. Vehicle consumption, on the other hand, is affected neither by rearing nor by rh5-HTTLPR genotype. Although our limited sample size dictates that we interpret our data with caution, it appears as if maternal input provides a buffering function, such that MR animals with the l/s genotype, unlike PR l/s animals, are not at risk for high alcohol preference or intake. This is reminiscent of the stress × biological risk diathesis model that was proposed in early theories of psychopathology. What this particular study provides is an updated approach to understanding psychopathology and its treatment, explaining the basis for the observations that 2 individuals from similarly impoverished environments, but with different genetic backgrounds, show disparate developmental outcomes. It is also consistent with twin studies, which show that identical twins are not always concordant for psychopathology. Finally, our findings are in agreement with those of Caspi et al.,48 which demonstrate interactions between serotonin transporter gene promoter variation and stress in the pathogenesis of depression.

Previous studies in our laboratory have shown that PR female macaques have augmented corticotropin responses to alcohol and that elevated corticotropin levels persisted for weeks following discontinuation of the alcohol consumption study.49 In the present study, we have demonstrated that early experience and exposure to alcohol interact with one another, such that while there is no effect of rearing condition on alcohol consumption during initial exposures to alcohol, females subjected to stress early in life, especially those who are carriers of the s allele, demonstrate progressive increases in their levels of alcohol consumption, achieving levels that would be expected to produce blood alcohol concentrations in the range of 100 mg%-150 mg%.49 This is in contrast to the maximal alcohol intake observed among MR females, whose consumption would be predicted to produce blood alcohol concentrations in the range of 50 mg%, well lower than the legal level of intoxication. Corticosteroids are thought to influence hepatic expression of alcohol dehydrogenase50 and therefore could result in acceleration of the development of induced tolerance to alcohol with progressive exposures. Although in PR female macaques, differences in levels of corticotropin have been noted following exposures to alcohol, and there does not appear to be a differential effect of alcohol on total cortisol levels among these animals. We therefore do not think that this is a likely explanation for accelerated increases in rates of consumption among PR females.

Studies have shown that while alcohol acutely decreases glucocorticoid response element binding in the rat amygdala (which other studies suggest would, ultimately, produce anxiolysis),51 serotonin receptor antagonism prevents this from occurring.52 This suggests the potential for an interaction between the serotonin system, alcohol, and amygdalar reactivity during exposures to alcohol. Although, unlike rodents, primates are not thought to become sensitized to the positive reinforcing effects of drugs of abuse, they may, like humans, become sensitized to alcohol's negative reinforcing effects. Recently, we demonstrated there to be an interaction between rearing condition and rh5-HTTLPR genotype on LHPA-axis activation in female macaques.53 One potential mechanism that could explain the progressive increases in alcohol consumption observed in PR females (especially those carrying the s allele) is rapid sensitization to the negative reinforcing effects of alcohol. If it is the case that female PR l/s animals progressively increase their alcohol intake in order to alleviate the negative symptoms (ie, anxiety or dysphoria) associated with alcohol exposure, then in human populations, women with variation in the serotonin transporter gene promoter who are also exposed to early-life stress may be particularly vulnerable to alcoholism. The influences of early experience and chronic stress are particularly relevant with regard to type I alcoholism,52 which is related to anxiety and is the more common type of alcoholism among women. It is also of interest that the s allele has, in several instances, been associated with suicide ideations among type 1 alcoholics, suggesting an interaction between alcohol intake and serotonin transporter gene variation in the etiology of severe depressive symptoms.54 In addition, there are known interactions between 5-HTTLPR and stress in the incidence of depression.55 It may be that alcohol preference in PR l/s females is reflective not only of altered serotonin release following exposure to alcohol but a predisposition to anxiety both independent of and in relation to alcohol consumption.

Traits characteristic of type 1 and type 2 alcoholism are thought to relate to dysregulated central nervous system serotonin functioning. To the extent that they generalize to humans, our findings suggest that the pathogenesis of alcohol dependence has its genetics, at least in part, in the interacting influence of early deleterious rearing experience and genetic factors. The similarity of humans and rhesus monkeys in genetic variation of the serotonin transporter gene promoter region as well as serotonin-mediated behavioral deficits suggest that the nonhuman primate model may have value for determining whether genetic variation may be used to identify or develop appropriate pharmacotherapies for the treatment of serotonin-related disorders, including alcoholism. It also allows us to observe behavioral patterns, for example, patterns of alcohol consumption during adolescence, that may lead to susceptibility, pathogenesis, and progression of alcohol-related disorders.

One major limitation in the treatment of addiction is the inability to restore the addicted brain to its predicated state. Early-life stress can cause persistent changes in the neuroendocrine stress axis and serotonin system, both of which are implicated in alcohol-induced allostatics and allostatic load in the brain.55 Since activation of the neuroendocrine stress axis and dysregulated serotonin neurotransmission are thought to be factors that predispose individuals to alcohol withdrawal, and therefore depression, dysphoria, and relapse, it is possible that combination therapies that both regulate the serotonin system and prevent overactivity of the neuroendocrine stress axis would not only help to prevent progression
of alcoholism and related disorders but may also be effective in returning the alcoholic brain to an earlier allostatic set point. By learning more about the interactions between genes, early experience, and alcohol intake in the nonhuman primate, we may better be able to design combination therapies for preventing and treating alcoholism.

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