

Binge alcohol drinking is associated with GABA_A α2-regulated Toll-like receptor 4 (TLR4) expression in the central amygdala

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Binge drinking (blood-alcohol levels ≥ 0.08 g% in a 2-h period), is a significant public health burden in need of improved treatment. Gene therapy may offer beneficial alternatives to current psychosocial and pharmacotherapeutic interventions, but identification of the target genes is a clinical challenge. We report that a GABA_A α2 siRNA vector (pHSVsiLA2) infused into the central nucleus of the amygdala (CeA) of alcohol-preferring (P) rats caused profound and selective reduction of binge drinking associated with inhibition of α2 expression, decreased GABA_A receptor density, and inhibition of Toll-like receptor 4 (TLR4). CeA infusion of a TLR4 siRNA vector (pHSVsiTLR4a) also inhibited binge drinking, but neither vector functioned when infused into the ventral pallidum. Binge drinking was inhibited by a GABA_A α1 siRNA vector (pHSVsiLA1) infused into the ventral pallidum, unrelated to TLR4. The vectors did not alter sucrose intake and a scrambled siRNA vector was negative. The data indicate that GABA_A α2-regulated TLR4 expression in the CeA contributes to binge drinking and may be a key early neuroadaptation in excessive drinking.

alcoholism | HSV vector | reinforcing effects | ethanol | innate immunity

Approximately 30% of the current drinkers in the United States drink alcohol excessively, a condition that kills ~75,000 people annually (1). Binge drinking (blood-alcohol level ≥ 0.08 g% in a 2-h period) is one form of excessive drinking (2) that is related to impulsivity and anxiety (3, 4), and represents a particularly problematic and hazardous form of excessive alcohol intake (5, 6). Gene therapy may offer a beneficial alternative to psychosocial and pharmacotherapeutic interventions, but a clinical challenge is the identification of the relevant target gene or genes. The GABA_A receptors are an established molecular target for excessive drinking (7). Microinfusion of α1-preferring ligands into the ventral pallidum (VP) (8), a locus containing the highest concentrations of α1 subunits in the reward circuitry (9), selectively regulated excessive drinking. Indeed, the VP is an important forebrain substrate receiving GABAergic projections from neurons of the extended amygdala (10), and it has been implicated in effort-related decision making pertaining to drug abuse (11). Marked reductions in alcohol drinking were also seen in α1 knock-out mice (12). However, adaptive changes in gene expression preclude final conclusions (13), and evidence of an association between the α1 subunit and alcohol drinking is relatively scant in humans (14). In contrast, human clinical linkage studies support a role for the α2 subunit in alcohol dependence, potentially reflecting the role of α2 in anxiety, impulsivity, and cognition (4, 15, 16). Unfortunately, compelling preclinical data to support the involvement of the α2 subunit in the control of excessive drinking are not currently available (13, 17).

The CeA is a major component of the extended amygdala that has been implicated in early neuroadaptations associated with

excessive drinking (18). Like the VP, the central nucleus of the amygdala (CeA) comprises GABA_A receptors that, when activated by ligand binding, suppress excessive drinking (19, 20). However, in contrast to the VP, the CeA comprises primarily α2 subunits (10) hypothesized to regulate states of emotionality [like fear and anxiety (16, 10)], which may be salient in the initiation of alcohol drinking (21). In so far as the anxiogenic response to alcohol withdrawal is a powerful motivator of excessive drinking (18), the α2 subunits may also be salient in regulating the negative affective states of alcohol dependence. However, the direct role of the α1 and α2 subunits in regulating binge drinking and their downstream molecular pathways are unknown.

Our studies follow on recent findings that: (i) proinflammatory cytokines/chemokines are involved in alcohol-related neurodegeneration (22), (ii) increased cytokine activity causes an adaptive change that supports sensitization of ethanol withdrawal-induced anxiety linked to GABA_A-receptors (23), and (iii) the innate immunity receptor Toll-like receptor 4 (TLR4) regulates proinflammatory cytokine responses (24) and has a pivotal role in alcohol-induced neuroinflammation and brain damage (25, 26). We report that the vulnerability to initiate/sustain binge alcohol drinking is associated with a GABA_A α2-TLR4 pathway in the CeA and GABA_A α1 in the VP. To the extent of our knowledge, this article is unique in reporting that an innate immunity receptor functions downstream of GABA_A receptors in the brain and is associated with the vulnerability to engage in binge alcohol drinking, providing a paradigm shift in the alcohol field.

Results

Infusion of the pHSVsiLA2 Vector into the CeA Inhibits Binge Drinking. We studied alcohol-preferring (P) rats, an established model of human alcohol abuse, recently shown to engage in National Institute on Alcohol Abuse and Alcoholism-defined effort-related (operant-lever) “binge” drinking (27, 28) (*SI Discussion*). Compared with nonalcohol-preferring (NP) rats, P rats have elevated levels of GABA_A subunits that are associated with excessive drinking in humans, notably α1 in the VP and both α1 and α2 in the CeA (*Fig. S1*). To examine whether these neuroanatomical site-specific elevations are associated with binge drinking, we used the siRNA technology that specifically inhibits one gene at

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one site (29). The siRNAs and the construction and specificity of the HSV-based vectors (amplicons) used to deliver them are described in *SI Materials and Methods* (Figs. S1 and S2, and Table S1). The vectors specifically inhibit their cognate targets and are not toxic. They do not cause loss of body weight or alter general activity levels in the P rats (Fig. S4), and do not induce cell death/apoptosis in intrastratially infused mice (Fig. S5). The vectors transduce neurons at the injection sites and do not traffic to distant brain areas as determined by EGFP visualization at 72 h after infusion. This result is shown in Fig. 1A and B for one of the nine CeA sites given pHSVsiLA2. Most EGFP⁺ cells likely contain GABA_A receptors, because the $\alpha 2$ and $\alpha 1$ subunits are highly expressed at these sites (30). Similar results were obtained for all of the vectors and in all examined neuroanatomical sites.

To initiate binge drinking, we used the operant drinking-in-the-dark-multiple-scheduled-access (DIDMSA) protocol developed by the Integrative Neuroscience Initiative on Alcoholism (INIA-West) (27, 28). Using this protocol, blood-alcohol levels were ≥ 110 mg/dL. Rats trained to binge on sucrose (0.1% wt/vol) on a similar schedule were studied in parallel. Cohorts of P rats ($n = 5-8$ per group) trained to self-administer alcohol were randomly given by cohort, pHSVsiLA2 ($\alpha 2$ -specific) or pHSVsiNC (scrambled control) into the CeA by bilateral stereotaxic infusion. After 3 d, during which the animals were allowed to recover from the stress of surgery, they were given the opportunity to engage in alcohol or sucrose drinking and examined daily for 15 d (Fig. 1C). On days 3 to 6 after surgery, pHSVsiLA2-infused rats had virtually no alcohol intake. Thereafter, drinking increased with time, returning to the original presurgery levels on day 13 after infusion. This finding is in direct contrast to rats given pHSVsiNC who evidenced minimally reduced alcohol drinking only during the first 3 d after surgery. Throughout the next 14 d of follow-up, the pHSVsiNC-treated rats displayed baseline (presurgery) levels of alcohol drinking (Fig. 1C) and pHSVsiLA2 did not decrease sucrose (Fig. 1D) or water (Fig. S64) consumption. Significantly, pHSVsiLA2 failed

to alter alcohol responding when given into the VP (Fig. 1E) or nucleus accumbens (NAcc) (Fig. S6B), both of which are neuroanatomical control loci. The data indicate that pHSVsiLA2 has both reinforcer and neuroanatomical specificity when infused into the CeA, suggesting that $\alpha 2$ overexpression at this site promotes alcohol drinking vulnerability, at least in P rats.

Infusion of pHSVsiLA1 into the CeA Does Not Inhibit Binge Drinking.

Because $\alpha 1$ expression is also elevated in the CeA from P rats (Fig. S1), we wanted to know if infusion of the $\alpha 1$ -specific vector pHSVsiLA1 can similarly reduce binge drinking. Cohorts of P rats ($n = 5-9$ per group) trained to self-administer alcohol under the "binge operant-lever press model" (27, 28) were randomly given by cohort pHSVsiLA1 or pHSVsiNC into the CeA and examined for alcohol consumption. Except for the first 3 d after surgery, when animals were still recovering from surgery, alcohol drinking was similar to that seen before surgery for both the pHSVsiLA1- and pHSVsiNC-infused rats (Fig. 1G). The data indicate that pHSVsiLA1 infusion into the CeA does not inhibit binge drinking, suggesting that $\alpha 1$ overexpression in the CeA does not contribute to the alcohol-drinking vulnerability of P rats.

Association of pHSVsiLA2-Mediated Decreased Drinking with $\alpha 2$ Inhibition in the CeA.

To examine whether pHSVsiLA2-mediated inhibition of binge drinking is a result of $\alpha 2$ protein inhibition, CeA tissues were collected at 3 and 15 d after infusion, when alcohol drinking was respectively ablated or restored to presurgery levels, and protein extracts were immunoblotted with $\alpha 2$ -specific antibody. The blots were stripped and reprobed with $\alpha 1$ -specific antibody to control for potential compensatory effects. CeA tissues from animals given pHSVsiNC or PBS, whose drinking was not reduced, were studied in parallel and served as controls. A dramatic decrease in the levels of $\alpha 2$ was caused by pHSVsiLA2 72 h after infusion, when alcohol drinking was ablated, but expression was restored at 15 d after infusion (Fig. 2A), when alcohol drinking was back to presurgery levels. The dura-

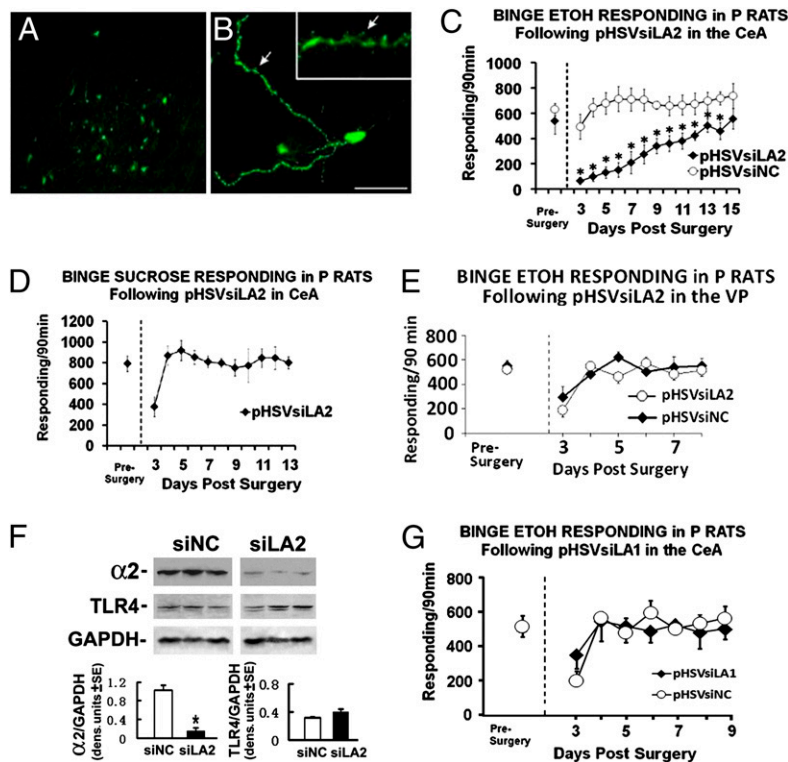


Fig. 1. CeA-delivered pHSVsiLA2, but not pHSVsiLA1, inhibits binge alcohol drinking. (**A** and **B**) A group of infected cells located near one of the pHSVsiLA2 injection sites (**A**) and an individual EGFP⁺ neuron (**B**). (*Inset*) Arrows show presence in processes and within individual spines. (Scale bar represents 250 μ m in **A** and 25 μ m in **B**, respectively.) (**C**) Binge alcohol (10% vol/vol) responding in P rats on an FR-4 schedule during presurgery (5 d), and after pHSVsiLA2 infusion into the CeA. The asterisks represent significance for pHSVsiLA2 ($n = 8$) compared with pHSVsiNC ($n = 5$) and presurgery controls using Tukey and Dunnett's test, respectively, after significant group \times session ANOVA [$F_{(7,56)} = 3.34$, $P < 0.034$]. (**D**) Binge sucrose (0.1% wt/vol) responding in P rats on an FR-4 schedule during the presurgery (5 d) and after pHSVsiLA2 ($n = 4$) infusion in the CeA. Except for the initial postsurgery day, pHSVsiLA2 did not alter binge sucrose responding ($P > 0.05$). (**E**) Binge alcohol responding in P rats on an FR-4 schedule during the presurgery (5 d), and after pHSVsiLA2 ($n = 5$) or pHSVsiNC ($n = 9$) infused into the VP. Except for the initial postsurgery day, pHSVsiLA2 did not alter binge alcohol responding ($P > 0.05$). (**F**) Extracts of VP micropunches, collected 72 h after pHSVsiLA2 or pHSVsiNC infusion, were immunoblotted with antibodies to $\alpha 2$, TLR4, or GAPDH used as loading control. The blots were stripped and reprobed with antibodies and results are expressed as densitometric units \pm SEM. The levels of $\alpha 2$, but not TLR4, were significantly lower in pHSVsiLA2 than pHSVsiNC rats ($*P < 0.001$ by ANOVA). (**G**) Binge alcohol responding in P rats given pHSVsiLA1 ($n = 5$) or pHSVsiNC ($n = 9$) into the CeA. Except for the initial postsurgery day, pHSVsiLA1 did not alter alcohol responding ($P > 0.05$). (See *SI Materials and Methods* for blood alcohol levels and additional statistical details for C–G.)

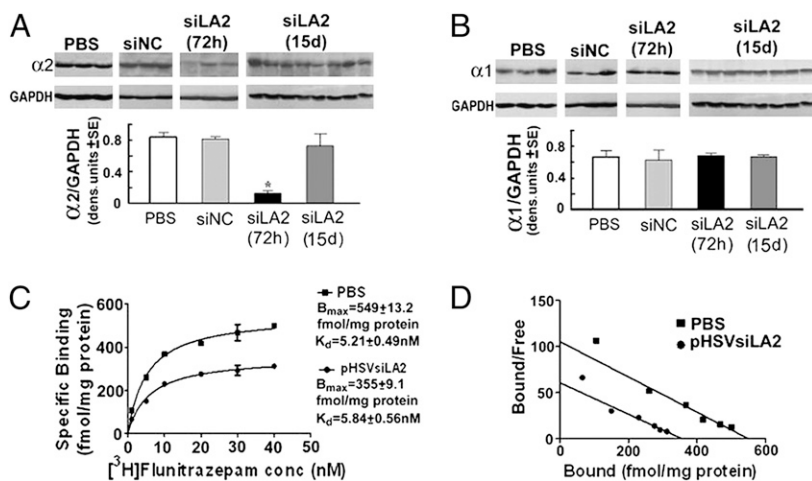


Fig. 2. Inhibition of $\alpha 2$ expression and receptor density in the CeA by pHSVsiLA2. Cohorts of P rats trained to binge on alcohol as in Fig. 1 were microinfused with PBS, pHSVsiNC, or pHSVsiLA2 into the CeA and micropunches were collected at 72 h or 15 d. Protein extracts were immunoblotted with antibodies to $\alpha 2$ (A), $\alpha 1$ (B), or GAPDH control. The blots were stripped between antibodies and results expressed as densitometric units \pm SEM. pHSVsiLA2 inhibited $\alpha 2$, but not $\alpha 1$, at 72 h ($*P < 0.001$ by ANOVA). Inhibition was not seen for pHSVsiNC and it was lost by day 15 after infusion of pHSVsiLA2 ($P > 0.05$). (C and D) [3 H]flunitrazepam binding in P rats microinfused in the CeA with PBS ($n = 10$) or pHSVsiLA2 ($n = 10$) 72 h postinfusion. Significant differences ($P < 0.0001$) were seen by scatchard analysis (C) and saturation isotherm (D). (See *SI Materials and Methods* for additional statistical details for A–D.).

tion of the inhibitory effect likely reflects that of siRNA integrity/availability and the resulting posttranscriptional gene silencing, as shown for pHSVsiLA1 in Fig. S7. This finding is consistent with previous reports for other unrelated siRNAs (31); $\alpha 2$ was not inhibited in animals given pHSVsiNC (Fig. 2A), and there were no compensatory effects on $\alpha 1$ expression, as evidenced by similar levels of $\alpha 1$ in animals given PBS, pHSVsiLA2, or pHSVsiNC (Fig. 2B). Significantly, pHSVsiLA2 inhibited $\alpha 2$ expression also when given into the VP (Fig. 1F), suggesting that its failure to inhibit binge drinking when given at this site (Fig. 1E) reflects the absence or lack of function of a downstream factor that is operative in the CeA.

Inhibition of Receptor Density in the CeA by pHSVsiLA2. The effect of pHSVsiLA2 in radioligand [3 H]flunitrazepam receptor binding was assayed, as previously described (32). [3 H]flunitrazepam binding was reduced by pHSVsiLA2 relative to the control condition (PBS-treated rats). Saturation isotherm showed significant differences in specific binding between the two groups. The binding (B_{max}) was reduced from 549 ± 13.2 fmol/mg protein in the control group to 355 ± 9 fmol/mg protein in the pHSVsiLA2-treated animals with no significant change in affinity [K_d] (control, 5.21 ± 0.49 nM; pHSVsiLA2-treated, 5.84 ± 0.56 nM) (Fig. 2C and D). Although flunitrazepam detects $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ receptor subunits (33), we conclude that the majority of the involved receptors are $\alpha 2$, because: (i) the CeA in P rats contains primarily $\alpha 2$ and $\alpha 1$ receptor subunits and, (ii) $\alpha 1$ expression was not altered by pHSVsiLA2. The data support the interpretation that the ability of pHSVsiLA2 to inhibit binge drinking is a result of receptor downregulation.

Inhibition of TLR4 Expression in the CeA, but Not VP, by pHSVsiLA2. We considered the possibility that the putative downstream factor that contributes to the $\alpha 2$ effect in the CeA is TLR4, because: (i) cytokines were implicated in alcohol-mediated brain damage potentially linked to GABA $_A$ -receptors (22, 23), (ii) TLR4 signaling was associated with neuroinflammation and brain damage (25, 26), and (iii) alcohol up-regulates genes in the Toll-innate immunity pathway in *Drosophila* (34). Cohorts of naive P rats ($n = 3$ –4 per group) were given pHSVsiLA2 or pHSVsiNC in the CeA, and tissues collected 72 h after infusion were immunoblotted with antibodies to $\alpha 2$ or TLR4. Antibody to another innate immunity receptor, CCR2, served as a control. There was a significant decrease in the levels of both $\alpha 2$ and TLR4 caused by pHSVsiLA2, but expression was not altered by pHSVsiNC (Fig. 3A). The expression of CCR2 did not decrease because of pHSVsiLA2 (Fig. 3A) and TLR4 expression was not reduced by pHSVsiNC. TLR4 inhibition is associated with the

ability of pHSVsiLA2 to inhibit binge drinking and is not an off-target effect of pHSVsiLA2, because pHSVsiLA2 did not inhibit TLR4 expression in RAW264.7 cells (Fig. S3B) nor when infused into the VP (Fig. 1F), a site at which it does not decrease binge drinking (Fig. 1E).

To further examine the relationship between the $\alpha 2$ -regulated TLR4 and binge drinking, a second series of experiments asked whether TLR4 is also inhibited by pHSVsiLA1, which does not affect binge drinking when infused into the CeA. Cohorts of naive P rats ($n = 3$ per group) were given PBS, pHSVsiLA1, or pHSVsiNC in the CeA and tissues collected 72 h after infusion were immunoblotted with antibodies to $\alpha 1$, $\alpha 2$, TLR4, or CCR2,

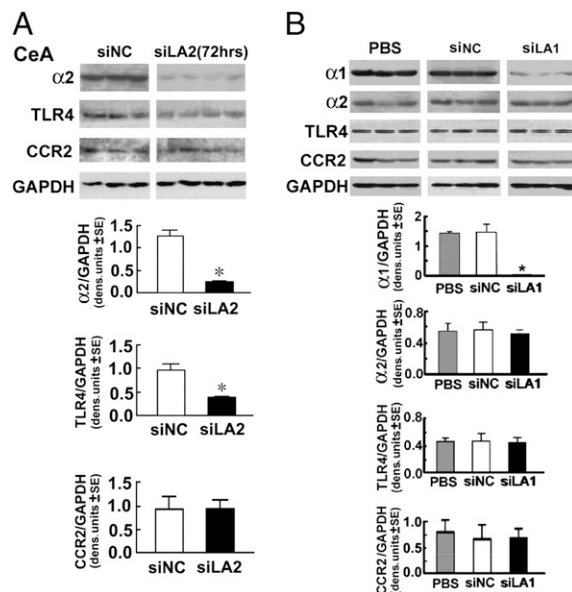


Fig. 3. CeA-delivered pHSVsiLA2, but not pHSVsiLA1, inhibits TLR4 expression. (A) Micropunches from the CeA of naive P rats microinfused with pHSVsiNC or pHSVsiLA2 collected 72 h after infusion were immunoblotted with antibodies to $\alpha 2$, TLR4, or CCR2 using GAPDH control. Both $\alpha 2$ and TLR4 were significantly lower in pHSVsiLA2 than pHSVsiNC rats ($*P < 0.001$ by ANOVA). (B) Micropunches of the CeA from naive P rats microinfused with PBS, pHSVsiNC, or pHSVsiLA1 were collected 72 h after infusion and immunoblotted with antibodies to $\alpha 1$, $\alpha 2$, TLR4, or CCR2 using GAPDH control; $\alpha 1$ was significantly lower in pHSVsiLA1 than PBS or pHSVsiNC-treated rats ($*P < 0.001$ by ANOVA). The levels of $\alpha 2$, TLR4, and CCR2 were similar ($P > 0.05$). (See *SI Materials and Methods* for additional statistical details for A and B.).

used as control. A significant reduction in the expression of $\alpha 1$, but not $\alpha 2$, TLR4, or CCR2, was caused by pHSVsiLA1, and pHSVsiNC was negative (Fig. 3B). Because pHSVsiLA1 does not inhibit binge drinking when infused into the CeA, the data implicate TLR4 as a downstream component of an $\alpha 2$ pathway in the CeA that regulates binge drinking.

CeA-Delivered pHSVsiTLR4a Selectively Inhibits Binge Alcohol Drinking. To confirm that TLR4 in the CeA contributes to the vulnerability to engage in binge drinking, cohorts of P rats trained to self-administer alcohol were randomly given, by cohort, pHSVsiTLR4a ($n = 10$) or pHSVsiNC ($n = 8$) into the CeA. After 3 d, during which the animals were allowed to recover from the stress of surgery, alcohol drinking was examined daily for 12 d. We found that pHSVsiTLR4a inhibited alcohol drinking, with maximal reduction seen on days 3 to 6 post-infusion. Drinking returned to presurgery levels on day 11 after infusion. In contrast, animals given pHSVsiNC evidenced minimally reduced alcohol drinking and only on days 3 to 5 after surgery, likely related to surgical stress (Fig. 4A). Binge sucrose-motivated responding was not altered (Fig. 4B). Reflecting its effect on binge drinking, pHSVsiTLR4a inhibited TLR4 protein expression on day 3, but not day 15 postinfusion (Fig. 4C). The data indicate that pHSVsiTLR4a treatment has both neuroanatomical and reinforcer specificity, supporting the interpretation that TLR4 is involved in the CeA effect on binge

drinking. We conclude that the TLR4 effect is neuronal, because pHSVsiTLR4a localized in neurons (Fig. 4D). Significantly, studies of P rats randomly given, by cohort, pHSVsiTLR4a ($n = 7$) or pHSVsiNC ($n = 8$) into the VP indicated that binge drinking was not altered by pHSVsiTLR4a given at this site (Fig. 4E). This finding is consistent with the failure of pHSVsiLA2 to inhibit binge drinking when given into the VP and suggests that the $\alpha 2$ contribution to binge drinking is through a TLR4-encompassing pathway that is not operative in the VP.

VP-Infused pHSVsiLA1 Inhibits Binge Drinking Unrelated to TLR4 Expression. Having seen that $\alpha 1$ is overexpressed in the VP from P rats (Fig. S1), we wanted to know whether it contributes to the vulnerability to engage in binge drinking. Cohorts of P rats ($n = 4$ –10 per group) trained to self-administer alcohol under the “binge operant-lever press model” (27, 28) were randomly given, by cohort, pHSVsiLA1, pHSVsiNC, or PBS into the VP and examined for alcohol consumption. After 3 d of recovery from the stress of surgery, alcohol drinking was examined daily for 13 d. Alcohol drinking was significantly decreased in animals given pHSVsiLA1, with maximal inhibitory levels seen on days 3 to 5 after infusion and returned to the presurgery levels on day 13 after infusion (Fig. 5A). This finding is in direct contrast to rats given PBS (Fig. 5A and B) or pHSVsiNC (Fig. 5B), who evidenced reduced alcohol drinking only during the first 3 to 4 d after surgery, likely related to the recovery from the surgical

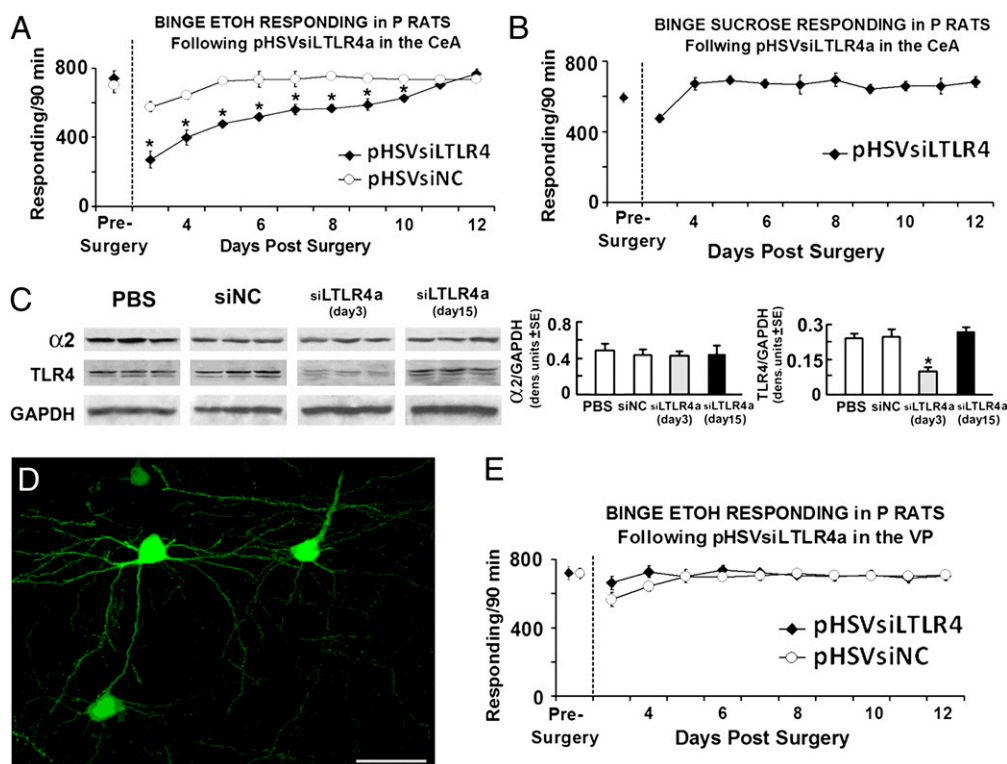


Fig. 4. CeA-delivered pHSVsiTLR4a selectively inhibits binge alcohol drinking. (A) Binge alcohol responding in P rats on an FR-4 schedule during the presurgery (5 d), and after pHSVsiTLR4a infusion into the CeA. The asterisk represents significance for pHSVsiTLR4a ($n = 10$) compared with pHSVsiNC ($n = 8$) and the presurgery control using the Tukey and Dunnett's test, respectively, following significant group [$F_{(1,10)} = 15.46, P < 0.003$] and session [$F_{(10,10)} = 3.42, P < 0.032$] effects. (B) Binge sucrose responding in P rats ($n = 5$) on an FR-4 schedule during the presurgery (5 d) and after pHSVsiTLR4a infusion into the CeA. Except for the initial postsurgery day, pHSVsiTLR4a did not significantly alter binge sucrose responding ($P > 0.05$). (C) Cohorts of binged P rats were microinfused with PBS, pHSVsiNC, or pHSVsiTLR4a into the CeA and micropunches collected 72 h or 15 d after infusion. Protein extracts were then immunoblotted with antibodies to $\alpha 2$, TLR4, or GAPDH control and blots stripped between antibodies and expressed as densitometric units \pm SEM. pHSVsiTLR4a inhibited TLR4 at day 3, but not day 15 relative to PBS and pHSVsiNC ($P > 0.05$); pHSVsiTLR4a also failed to alter the $\alpha 2$ protein ($P > 0.05$). (D) Group of EGFP⁺ neurons near one of the pHSVsiTLR4a injection sites in the CeA. (Scale bar, 25 μm .) (E) Binge alcohol responding in P rats on an FR-4 schedule during the presurgery (5 d), and after pHSVsiTLR4a ($n = 7$) and pHSVsiNC ($n = 8$) infused into the VP; pHSVsiTLR4a did not alter binge alcohol responding in the VP [$F_{(1,10)} = 2.78, P > 0.05$]. (See *SI Materials and Methods* for blood alcohol levels and additional statistical details for A–C and E.)

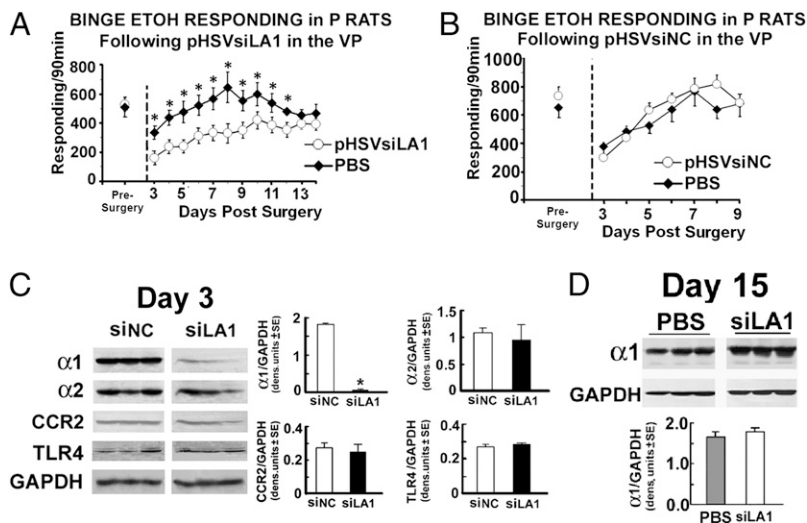


Fig. 5. VP-delivered pHSVsiLA1 inhibits binge alcohol drinking unrelated to TLR4. (A) Binge alcohol responding in P rats on an FR-4 schedule during the presurgery (5 d), and after pHSVsiLA1 infusion into the VP. The asterisk represents significance for pHSVsiLA1 compared with PBS and the presurgery control using the Tukey and Dunnett's test, respectively, following significant group [$F_{(1, 12)} = 50.31, P < 0.0001$] and session [$F_{(12, 182)} = 3.151, P < 0.0004$] effects. (B) Binge alcohol responding in P rats on an FR-4 schedule for presurgery (5 d), and after pHSVsiNC or PBS microinfused into the VP. Both displayed a similar profile of effects ($P > 0.05$). (C and D) Cohorts of P rats trained to binge on alcohol were microinfused with pHSVsiNC or pHSVsiLA1 into the VP and tissues collected at 72 h (C) or 15 d (D) were immunoblotted with antibodies to $\alpha 1$, $\alpha 2$, CCR2, or TLR4 using GAPDH as loading control. Results are densitometric units \pm SEM. pHSVsiLA1 inhibited $\alpha 1$ at day 3 ($P < 0.05$), but not 15 d postinfusion ($P > 0.05$). Expression of $\alpha 2$, CCR2, and TLR4 was not inhibited ($P > 0.05$). (See *SI Materials and Methods* for blood alcohol levels and additional statistical details for A–D.)

stress. We found that pHSVsiLA1 also failed to alter alcohol responding when given into the NAcc (Fig. S6C). Immunoblotting revealed a strong correlation between the inhibition of binge drinking and $\alpha 1$ expression (Fig. 5C), with $\alpha 1$ levels returning to the original presurgery levels by day 15 (Fig. 5D). The expression of $\alpha 2$ and TLR4 was not altered (Fig. 5C). The data indicate that $\alpha 1$ overexpression in the VP promotes alcohol drinking vulnerability, unrelated to TLR4.

Discussion

The GABA_A receptor is a well-recognized contributor to excessive alcohol drinking (4), but the development of effective therapies requires a better understanding of the specific receptor subunits, their brain loci, and the molecular pathways that transmit the signal and contribute to the regulation of the vulnerability to engage in excessive drinking. We report that the vulnerability to engage in binge drinking is differentially related to the GABA_A $\alpha 2$ and $\alpha 1$ subunits at distinct brain sites and is regulated by GABA_A $\alpha 1$ receptors in the VP and a GABA_A $\alpha 2$ -regulated pathway that includes TLR4 in the CeA. To the extent of our knowledge, this article is unique in reporting that an innate immunity receptor is under the control of the GABA_A receptors in the brain, and is associated with the vulnerability to engage in binge drinking.

To better evaluate the contribution of the differential GABA_A expression patterns to binge drinking, we used the siRNA technology that allows for specific inhibition of target genes at distinct brain sites. The specificity of our siRNA vectors is documented both in cultured cells and in the brains from microinfused rats and all experiments were done in parallel with an identical vector for scrambled siRNA (pHSVsiNC). The vectors are not toxic, as documented both at the gross and histologic levels, and they are expressed at the sites of stereotaxic delivery without evidence of trafficking to distant brain areas. Using these vectors, we found that pHSVsiLA2 infusion into the CeA caused a profound reduction in binge drinking that lasted 14 d and was not seen for pHSVsiNC. Inhibition of binge drinking was directly related to the inhibition of $\alpha 2$ expression through siRNA-mediated posttranscriptional gene silencing. Both were maximally reduced on days 3 to 6 postinfusion and increased with time thereafter returning to presurgery levels on day 14 postinfusion. This relatively long-lasting effect is consistent with previous reports for amplicon-delivered siRNAs (31). Loss of $\alpha 2$ expression (on day 3 postinfusion) had a profound and selective negative effect on the density of the GABA_A receptor (reduction to 65%), but additional studies are needed to define the quantitative relationship between the levels of $\alpha 2$ and the extent of binge drinking (EtOH responding/90 min)

and verify the effect of alcohol-induced changes and their siRNA modulation on receptor function.

Significantly, although $\alpha 1$ was also overexpressed in the CeA from P rats, its inhibition by infused pHSVsiLA1 did not reduce binge drinking. Conversely, drinking was inhibited by pHSVsiLA1-mediated inhibition of $\alpha 1$ expression in the VP, underscoring the different contributions of distinct GABA_A receptors at various brain regions to binge drinking and suggesting that they may function through distinct mechanisms. The NAcc, a well-established GABA_A alcohol-reward locus (18), was not associated with GABA_A $\alpha 1$ - or $\alpha 2$ -mediated binge drinking. The ability of pHSVsiLA2 to inhibit binge drinking when infused into the CeA was associated with inhibition of both $\alpha 2$ and TLR4 expression, but only $\alpha 2$ was inhibited by pHSVsiLA2 infusion into the VP. We conclude that in the CeA, TLR4 contributes to binge drinking downstream of $\alpha 2$, because the pHSVsiTLR4a vector infused at this site also inhibited binge drinking specifically associated with inhibition of TLR4, but not $\alpha 2$ expression. Further supporting the specificity of the $\alpha 2$ -TLR relationships, TLR4 was not inhibited by the CeA-infused pHSVsiLA1 vector—which does not inhibit binge drinking when given at this site—nor by pHSVsiLA1 infusion into the VP, where it inhibits binge drinking.

It seems reasonable to suggest that the alcohol-related connection between the $\alpha 2$ and TLR4 receptors is through elevated GABA production (35). GABA could stimulate the TLR4 responses directly (as a ligand) or indirectly via stimulation of chemokine/cytokine networks. This connection could have autocrine or paracrine effects that involve neurons and glial cells, all of which express TLR4. Previous studies have shown that ethanol induces neuronal damage involving TLR4-dependent microglial activation (25). However, the contribution of TLR4 to the initiation or sustaining of binge drinking, its regulation and mechanism, and the cells that are involved, are unknown. The convergence of our findings with existing topographical, neuroanatomical, neuropharmacological, and linkage studies suggests that binge drinking is a result of impaired neuronal inhibition caused by overactive $\alpha 2$ -containing GABA_A receptors in the CeA and $\alpha 1$ -containing GABA_A receptors of the VP. TLR4 contributes to binge drinking downstream of $\alpha 2$ in the CeA, but not VP, underscoring the relevance of TLR4 and specific neuroanatomical sites. The neuronal localization of pHSVsiTLR4a suggests that the $\alpha 2$ -TLR4 axis that contributes to binge drinking is neuronal. However, we cannot exclude the contribution of a small number of pHSVsiTLR4a-targeted glial cells that escaped detection, nor the potential crosstalk between the TLR4⁺ neurons and glial cells. An important, as yet unanswered question is: why does TLR4 contribute to

binge drinking only downstream of $\alpha 2$ and only in the CeA? It is possible that TLR4 contributes to binge drinking at other, as yet unstudied brain sites, potentially through distinct mechanisms implicated in the addiction cycle and involving different cell types. Ongoing studies are designed to address these questions and elucidate the potential contribution to binge drinking of chemokines/cytokines that were implicated in inflammation-related brain damage (22) and are TLR4-regulated (24). The amplicon-delivered siRNA technology provides an important tool to address these questions and may represent a promising therapeutic strategy in attenuating binge drinking behaviors.

Materials and Methods

Antibodies. The generation and specificity of the rabbit-derived GABA_A $\alpha 1$ and $\alpha 2$ antibodies have been previously described (30); they recognize amino acids 1 to 9 and 322 to 357 of the $\alpha 1$ and $\alpha 2$ proteins, respectively.

Small Interfering RNA Vectors. The siRNAs and the construction and specificity of the EGFP-containing vectors used for their delivery is described in *SI Materials and Methods*.

Immunoblotting. Tissue micropunches (300- μ m thick) were used in immunoblotting, done as previously described (36) and in further detail in *SI Discussion*.

Radioligand Binding. CeA membrane homogenates were prepared from adult male P rats and binding of [³H]flunitrazepam was determined by filtration assay (32). Briefly, homogenates were incubated (1 h, 4 °C) with varying concentrations of [³H]flunitrazepam (0.5–40 nM) in 50 mM Tris-HCl buffer

pH 7.4, containing 120 mM NaCl and 5 mM KCl. Nonspecific binding was determined with 100 μ M diazepam. Radioactivity was counted by liquid scintillation spectroscopy (Packard; TRI-CARB 2900 TR).

Binge Drinking. To initiate excessive alcohol drinking, we used the DIDMSA binge operant model developed by INIA-West (27, 28). The 90-min drinking sessions were conducted on a 5-d binge and 2-d withdrawal schedule that emulates human binge-drinking patterns (6). Rats consumed alcohol for 21 d before the presurgery phase.

Stereotaxic Procedures. Rats were anesthetized by intraperitoneal injection of nembutal (50 mg/kg) and positioned in a stereotaxic apparatus (8). Microinjection was in the CeA and VP (37). Each bregma level of each locus received 200 nL of PBS or amplicon (2.5×10^5 TU) delivered by calibrated glass micropipettes (~20- μ m tip) connected to a pneumatic pressure injector (Science Products GmbH). The Institutional Animal Care and Use Committee and Biosafety Committees, University of Maryland approved the procedures. See *SI Materials and Methods* for additional details.

Statistics. Data were analyzed by appropriate ANOVAs. Significant ANOVAs were followed by Dunnett and Tukey post hoc tests. Analyses were performed using the Stat Most 5.0 programs (Dataxiom Software Inc.).

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