

# Supporting Information

Liu et al. 10.1073/pnas.1019020108

## SI Discussion

**Animal Model of Human Binge Drinking.** The alcohol-preferring (P) rat line has been shown to fulfill most of the criteria for an animal model of human alcohol abuse to the satisfaction of the alcohol-research community (1, 2). The P rat will voluntarily consume alcohol to attain blood alcohol levels of 50 mg% to 200 mg%; lever press (i.e., work) for alcohol orally in concentrations of 10% to 40%; drink alcohol for its pharmacological effect; develop both tolerance and physical dependence following excessive intake, and upon removal, show signs of withdrawal following chronic consumption (2). Thus, with specific consideration to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth edition* criteria of human alcohol abuse, the P rat line represents an ideal model to emulate human binge drinking (1).

**P Rats Have Elevated Levels of GABA<sub>A</sub>  $\alpha$ 2 and  $\alpha$ 1 Subunits in the Central Nucleus of the Amygdala and Only  $\alpha$ 1 in the Ventral Pallidum.** P rats were examined for expression of the GABA<sub>A</sub>  $\alpha$ 2 and  $\alpha$ 1 subunits because they are associated with excessive drinking in humans (3, 4). Compared with nonalcohol-preferring (NP) rats, P rats showed elevated levels of  $\alpha$ 1 in the ventral pallidum (VP) (Fig. S1 A and B) and both  $\alpha$ 1 and  $\alpha$ 2 in the central nucleus of the amygdala (CeA) (Fig. S1 C and D), and similar results were obtained after initiation of alcohol drinking. The data indicate that the levels of both  $\alpha$ 2 and  $\alpha$ 1 are elevated in P rats, but at distinct neuroanatomical sites.

**Cells and Antibodies.** Rat insulinoma cells (RINm5F) constitutively express the GABA<sub>A</sub> receptor subunits  $\alpha$ 1 to -5,  $\beta$ 1 to -3,  $\gamma$ 1 to -3, and  $\delta$  (5). WS-1 cells are HEK 293 cells that constitutively express the rat GABA<sub>A</sub> receptor subunits  $\alpha$ 1,  $\beta$ 2, and  $\gamma$ 2 subunits (6). RAW 264.7 cells are mouse leukemic monocyte macrophage cells that express Toll-like receptor 4 (TLR4) (7). The cell lines were obtained from American Type Culture Collection. WS-1 and RAW 264.7 cells were cultured in DMEM with 4 mM L-glutamine (Invitrogen) and 10% FBS (Gemini). RINm5 cells were cultured in RPMI 1640 (Invitrogen) with 10% FBS. Vero 2.2 cells that express the HSV gene *ICP27* were the kind gift of Rachel Neve (McLean Hospital, Harvard Medical School, Boston, MA). These cells were cultured in DMEM-10% FBS and used as packaging cells for amplicon vector construction. Rat pheochromocytoma (PC12) cells were grown in DMEM with 10% heat-inactivated horse serum and 5% FBS. These cells were used for amplicon vector titration. The generation and specificity of the antibodies to the  $\alpha$ 1 and  $\alpha$ 2 GABA<sub>A</sub> receptor subunits were previously described (8, 9). TLR4 antibody (L-14, sc-16240) was purchased from Santa Cruz Biotechnology.

**HSV-1-Based Amplicon Vectors.** Therapies based on the use of replication-conditional HSV-1 vectors are particularly well suited for the treatment of CNS diseases and they are already moving toward clinical application (10, 11). HSV-1 amplicons are bacterial plasmids that contain two noncoding elements from HSV-1, an origin of DNA replication and a DNA packaging/cleavage signal. These elements allow replication and packaging into HSV-1 particles as a 150-kb concatamer. The amplicons do not express viral proteins and are not toxic. HSV-1 amplicons have a large transgene capacity that allows for the incorporation of various genes with therapeutic or regulatory functions and have been used for delivery of transgenes as well as siRNA. Numerous copies of the transgene sequences are packaged into one vector

particle, thereby allowing for high expression levels. The pHSVsi vector used to construct the amplicons was the kind gift of Cornel Fraefel (Institute of Virology, University of Zurich, Switzerland).

**Small Interfering RNAs and Their Inhibitory Activity.** Small interfering RNAs were designed to target distinct sequences within the  $\alpha$ 2 (Gene bank Entry No: NC\_005113.2) or  $\alpha$ 1 (Gene bank Entry No: NM\_183326.2) subunits of the rat GABA<sub>A</sub> receptor or the rat TLR4 gene (Gene bank Entry No: NC\_005104.2). A scrambled siRNA (NC) and a siRNA specific for the EGFP (7) served as controls. BLAST search against EST libraries was performed to ensure that no other gene was targeted. The siRNAs sequences targeted distinct genomic locations (target nucleotides) and had different inhibitory activity. Those sequences used in these studies are shown in Table S1. They were synthesized as 60-mer sense and antisense oligonucleotide templates (19  $\times$  2 nt specific to the targeted genes and 22 nt for restriction enzyme sites and hairpin structure). Synthesis was at the University of Maryland Biopolymer Core Facility and used the phosphoramidite (AB) technology. To confirm the ability of the siRNAs to inhibit cognate gene expression, cells were transfected with the siRNAs at a final concentration of 65 nM using the siPORT amine transfection agent (Ambion) according to the manufacturer's instructions, and extracts collected 72 h post-transfection were immunoblotted with the respective antibodies, as previously described (12–14).

**Packaging of siRNA Plasmids into HSV-1 Amplicon Vectors.** The pHSVsi vector used to generate the siRNA plasmids that are packaged into HSV-1 virions is schematically represented in Fig. S2. The vector expresses EGFP under the direction of the HSV-1 IE4/5 immediate-early promoter. The incorporation of EGFP allows for the titration of the vector stocks and the visualization of cell transduction in culture and in the CNS. The pSUPER plasmid, which contains the RNA polymerase III-dependent H1 promoter and well-defined start of transcription and termination signals, is used to generate a second transcription unit for the synthesis of siRNA. The siRNAs were inserted into the pHSVsi vector between the BglII and HindIII sites, downstream of the RNA polymerase III-dependent H1 promoter (Fig. S2) and packaged as previously described (11). Briefly, Vero 2–2 cells were transfected with the various plasmids (5  $\mu$ g) using FuGENE 6 transfection reagent (15  $\mu$ L) according to the manufacturer's instructions. After overnight incubation, the cells were infected with 6  $\times$  10<sup>5</sup> pfu of the HSV-1 *5dl1.2* helper virus, and the infected cells were collected 24 h later, when the cytopathic effect was at least 95%. Virus was released by freezing and thawing (virus P1) and further passaged in Vero 2–2 cells to increase the proportion of amplicons relative to helper virus. The P3 virus stock was clarified of cell debris by centrifugation and stored at –80 °C until further use. Virus titers were estimated in PC12 cells by counting the EGFP<sup>+</sup> cells 24 h after infection (absorbance 480 nm, emission 507 nm). For example, if 90% of the cells in a well infected with 2.5  $\mu$ L of virus are green, and if it is assumed that each EGFP<sup>+</sup> cell represents one infectious unit, then it can be inferred that the 2.5  $\mu$ L contained 2.7  $\times$  10<sup>6</sup> transducing units (TU) (i.e., 90% of 3  $\times$  10<sup>6</sup> cells plated in each well). Based on these calculations, the titers of our amplicon vectors were 1 to 2  $\times$  10<sup>9</sup> TU/mL. The amplicon vectors used in these studies are listed in Table S1.

**Function and Specificity of the HSV-1 Amplicon Vectors.** A series of experiments was done to examine the activity and specificity of the amplicon vectors. First, RINm5F cells were transduced with the pHSVsiLA2 or pHSVsiNC vectors at a multiplicity of infection of 3 to 5 TU per cell and  $\alpha 2$  expression was determined by immunoblotting 48 h after infection. To control for the possibility of compensatory activity, the blots were stripped and probed with antibody specific for  $\alpha 1$ . The results were quantified by densitometric scanning and expressed as percent of untreated control. Although pHSVsiLA2 inhibited  $\alpha 2$  expression (98–99%),  $\alpha 1$  expression was not affected. The pHSVsiNC was negative (Fig. S3A), indicating that pHSVsiLA2 has  $\alpha 2$ -specific inhibitory activity without  $\alpha 1$  compensatory effects. Second, RAW264.7 cells that do not express  $\alpha 2$ , but are positive for TLR4, were transduced with the pHSVsiLA2 or pHSVsiNC vectors at a multiplicity of infection of 3 to 5 TU per cell and expression of TLR4 and  $\alpha 2$  was determined by immunoblotting 48 h after transduction. pHSVsiLA2 did not inhibit TLR4 expression and pHSVsiNC was negative (Fig. S3B). The failure of pHSVsiLA2 to inhibit TLR4 confirms the specificity of the vector and is not a technical artifact, because TLR4 expression was inhibited in RAW264.7 cells transfected with the pHSVsiLTLR4a vector (Fig. S3C). In a final series of experiments, WS-1 cells were transduced with the pHSVsiLA1, pHSVsiNC, or pHSVsiEGFP vectors at a multiplicity of infection of 5 to 10 TU per cell and  $\alpha 1$  gene expression was determined by immunoblotting 48 h later. The  $\alpha 1$  expression was significantly reduced by pHSVsiLA1, but not pHSVsiNC or pHSVsiEGFP (Fig. S3D). The failure of pHSVsiNC to inhibit expression is not an artifact because of its failure to infect cells, because EGFP staining was similar in cells transduced with pHSVsiNC, pHSVsiLA2, pHSVsiLTLR4a, or pHSVsiLA1, and it encompassed 85% to 93% of the cells in the culture. Collectively, the data indicate that all the amplicon vectors used in these studies are specific for their respective cognate targets without any detectable compensatory activities.

**Behavioral Toxicity Measures.** To examine whether amplicons are toxic at the behavioral level, we examined body-weight gain and multiple parameters of locomotor behavior before and following amplicon infusions. Day 4 was selected as the initial postsurgery day, as it was devoid of factors related to recovery from the stress of the surgery itself.

**Body-weight measurements.** Rats ( $n = 8$  per group) were weighed twice weekly to determine if nonspecific effects of either the surgery or the amplicons could be observed on body-weight gain during the 2 wk of alcohol drinking. As shown in Fig. S4A, body-weight gains for the amplicon-treated rats were similar to those seen for the PBS control animals. Both groups were observed to nonsignificantly increase their postsurgical weight at 4 d post-treatment.

**Locomotor activity measurements.** Locomotor activity measurements (e.g., horizontal activity or ambulatory behavior) were recorded individually for 10 min in a Plexiglas chamber (42 cm  $\times$  42 cm  $\times$  30 cm) using a 16-beam infrared Digiscan Activity Monitoring System (Accuscan Electronics). Ambulatory behavior, measured by the total number of beam breaks in the horizontal plane (i.e., horizontal activity), is used as a sensitive measure of the motivational effects in rats (15). These experiments were conducted using previously published procedures (15). As shown in Fig. S4B, none of the amplicon treatments altered ambulation 4 d postsurgery relative to the PBS control. Thus, open-field behaviors of the two groups were similar. These data, taken along with the failure of the amplicon treatment to alter body-weight measures, suggest that treatment was not associated with overt behavioral toxicity effects.

**Amplicons Do Not Induce Apoptosis in the CNS Following Intrastriatal Delivery.** Intracranial HSV-1 injection causes fatal encephalitis

that is histologically characterized by cell death and apoptosis in ~35% to 45% of the cells (16). Therefore, apoptosis was used as a marker of amplicon-induced relatively rare histologic toxicity effects. Mice were given pHSVsiLA2, pHSVsiLA1, pHSVsiNC, or PBS ( $n = 5$  each) at 10 sites in the striatum ( $1.5 \times 10^5$  TU each) and followed for 100 d, as previously described (17). There was no evidence of physical or behavioral toxicity, and striatal sections collected at this time did not stain with antibody to activated caspase-3 (1–3% positive cells in all four study groups). This finding is in contrast to mice ( $n = 5$ ) given HSV-1 at a 10-fold lower dose ( $2 \times 10^4$  pfu) and at only one striatal site that died on days 5 to 11 postinjection and had 40% to 55% caspase-3p20<sup>+</sup> (activated caspase-3) cells in the striatum (Fig. S5). These data indicate that amplicons do not have gross or histologic toxicity related to the type of siRNA, their construction, or the dose and number of intracranial injections. EGFP tracking indicated that the amplicons did not traffic to brain regions distal from the microinjection site.

**Inhibitory Activity of the pHSVsiLA1 and pHSVsiLA2 Amplicon Vectors Is Specific for Alcohol and the Neuroanatomical Loci of Injection.** The extended amygdala has been suggested to be a key neurobiological circuitry in the binge-intoxication state of excessive drinking (18). The extended amygdala comprises the central nucleus of the CeA, shell of the nucleus accumbens (NAcc), and bed nucleus of the stria terminalis (19). The CeA and NAcc contain both  $\alpha 1$  and  $\alpha 2$  subunits (19), such that the NAcc represents an optimal neuroanatomical control locus for evaluating specificity of the pHSVsiLA2 and pHSVsiLA1 vectors. To test this interpretation, binge drinking was initiated using the operant methodology drinking-in-the-dark-multiple-scheduled-access [DIDMSA] protocol (1). Rats were adapted to a 12-h:12-h light/dark cycle and received three 30-min access periods to 10% alcohol spaced 1 h apart across the dark cycle for 3 mo. Animals received water during the 22.5-h nonalcohol drinking periods. The 90-min drinking sessions were conducted on a 5-d binge and 2-d withdrawal schedule that emulates human alcohol binge-drinking patterns (20). Evaluation of water intake and sucrose-reinforced responding was used to assess vector reinforcer specificity. In contrast to the reduction in alcohol responding (Fig. 1C), pHSVsiLA2 infusion into the CeA failed to significantly alter water consumption, such that intake was similar for rats given pHSVsiLA2 or pHSVsiNC (Fig. S6A). Both pHSVsiLA2 (Fig. S6B) and pHSVsiLA1 (Fig. S6C) also failed to alter binge alcohol drinking when given into the NAcc. Collectively, the data indicate that the pHSVsiLA2 and pHSVsiLA1 vectors evidence alcohol and brain-site specific activities.

**Duration of the Inhibitory Activity for Binge Alcohol Drinking Reflects That of Gene Silencing.** Small interfering RNA is easily degraded by enzymes in cells and tissues (reviewed in ref. 21), thereby limiting its availability to inhibit gene expression. Furthermore, its accumulation at the target site is extremely low after administration, underscoring the need for an effective delivery system to achieve gene silencing at the site of action. Previous studies have shown that siRNA delivered with an amplicon vector is capable of inhibiting target genes in vivo for at least 10 d (11). To examine the duration of target-gene inhibition by our vectors, rats trained to self-administer alcohol were randomly given by cohort, PBS, pHSVsiLA1, or pHSVsiNC into the VP ( $n = 6$ –10 per group) and examined for  $\alpha 1$  expression by immunoblotting at days 3, 10, and 15 postinfusion. These days respectively correspond to maximal inhibition of alcohol intake (day 3), partially restored drinking (day 10), and fully restored drinking (day 15) (Fig. 5A). The  $\alpha 1$  expression was identical in animals given pHSVsiNC or PBS. Relative to these groups,  $\alpha 1$  expression was maximally inhibited by pHSVsiLA1 on day 3 after infusion (Fig. S7A) and was fully restored on day 15 after infusion (Fig. S7C).

However, on day 10 after pHSVsiLA1 infusion, the levels of  $\alpha 1$  were significantly higher than those seen in rats given pHSVsiLA1 and examined on day 3 postinfusion, but still lower than those seen in the control groups (Fig. S7B). Similar results were obtained for pHSVsiLA2 and pHSVsiTLR4a. Collectively, the data indicate that the duration of gene silencing is  $\sim 2$  wk and it correlates with the effect of the amplicon-delivered siRNAs on binge alcohol drinking.

## SI Materials and Methods

**Stereotaxic Procedures.** Rats were anesthetized by intraperitoneal injection of nembutal (50 mg/kg) and positioned in a stereotaxic apparatus (22). The microinjection sites in the VP extend from +2.2 mm anterior to bregma to  $-0.8$  mm posterior to bregma, and from 0.5 mm to 3.0 mm lateral to the midline. The microinjection sites for the CeA extend from  $-1.60$  mm posterior to bregma to  $-3.3$  mm posterior to bregma, and from 3.0 mm to 4.5 mm lateral to the midline (23). Because amplicons do not diffuse over long distances, a single large injection would fail to cover the entire VP or CeA and likely result in a pressure lesion. Accordingly, we gave 9 or 13 small injections in each hemisphere spaced across the entire CeA or VP, respectively. Each site received 200 nL of PBS or amplicon ( $2.5 \times 10^5$  TU) delivered with a calibrated pulled glass micropipette ( $\sim 20$ - $\mu$ m tip) connected to a Picospritzer II pneumatic pressure injection apparatus (Science Products GmbH). Injections were over 30 s followed by a 1- to 2-min pause for tissue recovery before insertion of the pipette at the next site. Acrylic microbeads were used to confirm accuracy of the microinjection placement based on the Rat Brain Atlas (23). The Institutional Animal Care and Use Committee and Biosafety Committees of the University of Maryland approved the procedures.

**Immunoblotting.** Tissue micropunches (300- $\mu$ m thick) were used in immunoblotting. Lysis was in RIPA buffer [20 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate] with 1% phosphatase and protease inhibitor mixtures (Sigma) and total protein was determined by the bicinchoninic assay (Pierce). Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were exposed to antibody overnight at 4  $^{\circ}$ C (Cell Signaling) for 1 h at room temperature. Detection was with the ECL kit reagents (Amersham Life Science) and quantitation was by densitometric scanning with a Bio-Rad GS-700 imaging densitometer (13, 16).

**Blood-Alcohol Concentration and Statistical Analyses.** To emulate human binge drinking, binge drinking in P rats was initiated using the operant methodology DIDMSA protocol (1). Because blood-alcohol concentration (BAC) measurements are critical in defining human binge drinking (1), BAC was determined in duplicates at 30 and 90 min using previously described procedures (22). BACs taken at the 90-min period were used as the presurgery BAC measurements and are described below. In all cases, in Figs. 1, 4, and 5, as well as Fig. S6, BAC measurements exceeded  $\geq 80$  mg%/dL at the 90-min operant session. This BAC measurement is consistent with the National Institute on Alcohol Abuse and Alcoholism definition of binge alcohol consumption in humans (1, 20).

In addition to the information on BAC, additional statistical details are provided below to address the time-course effects of the amplicon vectors in the alcohol and sucrose drinking studies, as well as the immunoblot experiments. This information refers to the data presented in the main text. In all cases, post hoc analyses followed significant ANOVA. Specifically, both the Tukey and Dunnett's post hoc tests, which contain corrections for multiple comparisons, given the assumption of equal variances have been met between the two groups (24, 25), both post hoc tests were

used in the behavioral studies, but only the Tukey was used in the immunoblot analyses. The rationale for using the Tukey in both behavioral and immunoblot studies is because it has greater power than the other post hoc tests under most circumstances in detecting true and reliable differences between groups (24, 25). The Dunnett's test is similar to the Tukey test but is recommended only if a set of comparisons is repeatedly being made to one particular control group (25). The former test reflects the comparisons against the presurgery control baseline for the postsurgical days in the behavioral studies. Taken together, the additional statistical analyses, combined with the Tukey and Dunnett's post hoc tests, which both contain corrections for multiple comparisons, provide additional data on the time course of the long-term reduction effects of the amplicon vectors in the behavioral and immunoblot studies.

**Fig. 1: CeA-Delivered pHSVsiLA2, but Not pHSVsiLA1 Inhibits Binge Alcohol Drinking.** Fig. 1C shows binge alcohol (10% vol/vol) responding in P rats on an FR-4 schedule during the presurgery (5 d), and after pHSVsiLA2 infusion into the CeA. Presurgery BACs in the pHSVsiLA2 ( $n = 8$ ) and pHSVsiNC ( $n = 5$ ) groups were  $122 \pm 21$  and  $133 \pm 26$  mg%/dL, respectively. Two-way ANOVA revealed significant group [ $F_{(1,7)} = 42.05, P < 0.0001$ ], session [ $F_{(7,56)} = 2.82, P < 0.01$ ], and group  $\times$  session [ $F_{(7,56)} = 3.34, P < 0.034$ ] effects. The Tukey post hoc-test compared the pHSVsiLA2 and pHSVsiNC groups across postsurgical days 3 through 15, respectively. The Dunnett's post hoc-test compared the presurgery condition of the pHSVsiLA2 and pHSVsiNC groups against the postsurgical days 3 through 15 for the pHSVsiLA2 and pHSVsiNC groups, respectively. The asterisks in Fig. 1C represent significance for both the Tukey and Dunnett's comparisons. Fig. 1D shows binge sucrose (0.1% wt/vol) responding in P rats ( $n = 4$ ) on an FR-4 schedule during the presurgery (5 d) and after pHSVsiLA2 infusion in the CeA. Except for the initial postsurgery day (e.g., day 3) [ $F_{(11,60)} = 6.24, P < 0.001$ ], pHSVsiLA2 did not significantly alter binge sucrose responding. The Dunnett's post hoc-test confirmed significance for day 3 [ $P < 0.005$ ], but not days 4 to 13 [ $P \geq 0.05$ ]. Fig. 1E shows binge alcohol (10% vol/vol) responding in P rats given pHSVsiLA2 on an FR-4 schedule during the presurgery (5 d), and after pHSVsiLA2 ( $n = 5$ ) or pHSVsiNC ( $n = 9$ ) infused into the VP. Before surgery, BACs in the pHSVsiLA2 and pHSVsiNC groups were  $110 \pm 32$  and  $138 \pm 28$  mg%/dL, respectively. Except for the initial postsurgery day [ $F_{(6,83)} = 6.16, P < 0.001$ ], pHSVsiLA2 did not significantly alter binge alcohol responding. The Dunnett's post hoc-test, confirmed a significant difference for day 3 [ $P < 0.005$ ], but not days 4 to 8 ( $P \geq 0.05$ ). No group effects were observed [ $F_{(1,6)} = 1.66, P > 0.05$ ]. Fig. 1F shows that 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 between 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-treated rats [ $F_{(1,4)} = 61.54, P < 0.001$ ], [ $F_{(1,4)} = 0.97, P > 0.005$ ], ( $*P < 0.001$  by Tukey). Fig. 1G shows binge alcohol responding in P rats given pHSVsiLA1 ( $n = 5$ ) or pHSVsiNC ( $n = 9$ ) into the CeA. Before surgery, BACs in the pHSVsiLA1 and pHSVsiNC groups were, respectively,  $119 \pm 14$  and  $118 \pm 21$  mg%/dL. The pHSVsiLA1 treatment produced no group or interaction effect; however, the reduction on postsurgery day 1 resulted in a significant day effect [ $F_{(7,106)} = 3.74, P < 0.001$ ]. The Tukey test showed no difference between the pHSVsiLA1 and pHSVsiNC groups across days ( $P > 0.05$ ). The Dunnett's test showed no difference between pre- and postsurgery days 4 through 9 for the pHSVsiLA1 and pHSVsiNC groups ( $P > 0.05$ ).

**Fig. 2: pHSVsiLA2 Inhibits  $\alpha 2$  Expression and Receptor Density in the CeA.** Cohorts of P rats trained to binge on alcohol (10% vol/vol) as in Fig. 1 were microinfused with PBS, pHSVsiNC, or pHSVsiLA2 into the CeA and micropunches were collected from the right and left hemispheres at 72 h or 15 d after infusion. The tissues from the left and right hemispheres were pooled and protein extracts were immunoblotted with antibodies to  $\alpha 2$  (Fig. 2A),  $\alpha 1$  (Fig. 2B), or GAPDH used as loading control. The blots were stripped between antibodies and results are expressed as densitometric units  $\pm$  SEM. pHSVsiLA2 inhibited  $\alpha 2$ , but not  $\alpha 1$  at 72 h, resulting in a significant treatment effect [ $F_{(3, 13)} = 4.14, P < 0.029$ ]. The Tukey test confirmed the effects of pHSVsiLA2 at 72 h ( $*P < 0.001$ ) but not 15 d ( $P > 0.05$ ). Inhibition was not seen for pHSVsiNC ( $P > 0.05$ ). [ $^3\text{H}$ ]-flunitrazepam binding in P rats microinfused in the CeA with PBS ( $n = 10$ ) or pHSVsiLA2 ( $n = 10$ ) 72 h postinfusion. Significant differences were seen by scatchard analysis (Fig. 2C) and saturation isotherm (Fig. 2D) [ $F_{(3, 312)} = 107, P < 0.0001$ ], followed by Tukey post hoc test ( $*P < 0.001$ ).

**Fig. 3: CeA-Delivered pHSVsiLA2, but Not pHSVsiLA1, Inhibits TLR4 Expression.** Micropunches from the CeA of naive P rats microinfused with pHSVsiNC or pHSVsiLA2 and collected at 72 h after infusion were immunoblotted with antibodies to  $\alpha 2$ , TLR4, or CCR2 using GAPDH antibody as loading control. The levels of  $\alpha 2$  and TLR4, but not CCR2, were significantly lower in pHSVsiLA2- than in pHSVsiNC-treated rats, [ $F_{(1, 5)} = 89.54, P < 0.001$ ], [ $F_{(1, 5)} = 37.09, P < 0.002$ ], and [ $F_{(1, 5)} = 0.834, P > 0.002$ ], respectively (Fig. 3A). 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 antibody as loading control. The levels of  $\alpha 1$  were significantly lower in pHSVsiLA1- [ $F_{(2, 6)} = 81.45, P < 0.001$ ] than PBS- or pHSVsiNC-treated rats. The levels of  $\alpha 2$ , TLR4, and CCR2 were similar in all study groups. The Tukey test followed each significant ANOVA ( $*P \leq 0.05$ ) (Fig. 3B).

**Fig. 4: CeA-Delivered pHSVsiTLR4a Selectively Inhibits Binge Alcohol Drinking.** Binge alcohol (10% vol/vol) responding in P rats on an FR-4 schedule during the presurgery (5 d), and after pHSVsiTLR4a infusion into the CeA. Presurgery BACs in the pHSVsiTLR4 ( $n = 10$ ) and pHSVsiNC ( $n = 8$ ) groups were  $143 \text{ mg\%/dL} \pm 26$  and  $131 \text{ mg\%/dL} \pm 34$ , respectively. Two-way ANOVA revealed significant group [ $F_{(1,10)} = 15.46, P < 0.003$ ] and session [ $F_{(10, 10)} = 3.42, P < 0.032$ ] effects. The Tukey test showed a significant reduction in the pHSVsiTLR4a group compared with the pHSVsiNC group across postsurgery days 3 to 10 ( $P \leq 0.05$ ). The Dunnett's test showed a significant reduction in pHSVsiTLR4a treatment for postsurgery days 3 to 10 compared with the presurgery pHSVsiTLR4a condition ( $P \leq 0.05$ ). The asterisks in Fig. 4A represent significance for both the Tukey and Dunnett's comparisons. Binge sucrose (0.1% wt/vol) responding is shown in P rats ( $n = 5$ ) on an FR-4 schedule during the presurgery (5 d) and after pHSVsiTLR4a infusion in the CeA. Except for the initial postsurgery recovery day [ $F_{(10, 44)} = 2.54, P < 0.020$ ], the pHSVsiTLR4 treatment failed to significantly alter binge sucrose responding (Dunnett's

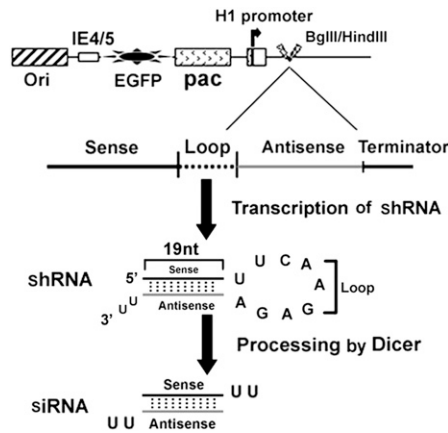
test,  $P > 0.05$ ) (Fig. 4B). Cohorts of P rats trained to binge on alcohol (10% vol/vol) were microinfused with PBS, pHSVsiNC, or pHSVsiTLR4a into the CeA and micropunches were collected from the right and left hemispheres at 72 h or 15 d after infusion. The tissues from the left and right hemispheres were pooled and protein extracts were immunoblotted with antibodies to  $\alpha 2$ , TLR4, or GAPDH used as loading control. The blots were stripped between antibodies and results are expressed as densitometric units  $\pm$  SEM. pHSVsiTLR4 inhibited TLR4 at day 3, but not at day 15 relative to the PBS and pHSVsiNC control groups, resulting in a highly significant treatment effect [ $F_{(3, 8)} = 23.64, P < 0.001$ ]. The Tukey test confirmed the pHSVsiTLR4 amplicon markedly reduced the TLR4 protein levels at 72 h postinfusion ( $*P < 0.001$ ). In contrast, the pHSVsiTLR4 amplicon failed to alter the  $\alpha 2$  protein [ $F_{(3, 8)} = 2.01, P > 0.05$ ] on day 3, or 15 postinfusion (Fig. 4C). A group of EGFP<sup>+</sup> neurons near one of the pHSVsiTLR4a injection sites in the CeA is shown (Fig. 4D). (Scale bar, 25  $\mu\text{m}$ .) Binge alcohol (10% vol/vol) responding in P rats on an FR-4 schedule during the presurgery (5 d), and after pHSVsiTLR4 ( $n = 7$ ) or pHSVsiNC ( $n = 8$ ) infusion into the VP. Before surgery, BACs in the pHSVsiTLR4a and pHSVsiNC groups were  $147 \text{ mg\%/dL} \pm 24$  and  $121 \text{ mg\%/dL} \pm 38$ , respectively. The pHSVsiTLR4a did not alter binge drinking in the VP. No significant treatment [ $F_{(1, 10)} = 2.89, P > 0.05$ ] or day [ $F_{(10, 10)} = 2.01, P > 0.05$ ] effects emerged (Fig. 4E).

**Fig. 5: VP-Delivered pHSVsiLA1 Inhibits Binge Alcohol Drinking Unrelated to TLR4.** Binge alcohol (10% vol/vol) responding in P rats on an FR-4 schedule during the presurgery (5 d), and after pHSVsiLA1 infusion into the VP. Presurgery BACs in the pHSVsiLA1 ( $n = 8$ ) and PBS ( $n = 5$ ) groups were  $132 \text{ mg\%/dL} \pm 26$  and  $116 \text{ mg\%/dL} \pm 32$ , respectively. Two-way ANOVA revealed significant group [ $F_{(1, 12)} = 50.31, P < 0.0001$ ] and session [ $F_{(12, 182)} = 3.151, P < 0.0004$ ] effects. The Tukey test confirmed that pHSVsiLA1 reduced binge responding for 3 to 12 d relative to the PBS control group ( $P \leq 0.05$ ). The Dunnett's test showed that the pHSVsiLA1 postsurgery days 3 to 12 were markedly reduced relative to the presurgery condition ( $P \leq 0.05$ ). The asterisk in Fig. 5A represents significance for both the Tukey and Dunnett's comparisons. Binge alcohol responding in P rats on an FR-4 schedule for presurgery (5 d), and after pHSVsiNC or PBS microinfusion into the VP. Before surgery, BACs in the pHSVsiNC ( $n = 4$ ) and PBS control ( $n = 4$ ) groups were  $122 \text{ mg\%/dL} \pm 21$  and  $133 \text{ mg\%/dL} \pm 26$ , respectively. Except for the initial postsurgery days [ $F_{(7, 48)} = 8.28, P < 0.001$ ], the pHSVsiNC- and PBS-treated groups displayed a similar profile of effects over days 5 through 9, with no significant effect of group [ $F_{(1, 14)} = 2.14, P > 0.05$ ] (Fig. 5B). Cohorts of P rats trained to binge on alcohol were microinfused with pHSVsiNC or pHSVsiLA1 into the VP and tissues collected 72 h (Fig. 5C) or 15 d (Fig. 5D) later were immunoblotted with antibodies to  $\alpha 1$ ,  $\alpha 2$ , TLR4, or CCR2 using GAPDH as loading control. Results are densitometric units  $\pm$  SEM pHSVsiLA1 inhibited expression of  $\alpha 1$  at day 3 [ $F_{(1, 4)} = 36.95, P < 0.001$ ], but not 15 postinfusion. Expression of  $\alpha 2$ , CCR2, and TLR4 was not inhibited. The Tukey test followed each significant ANOVA ( $*P \leq 0.01$ ).

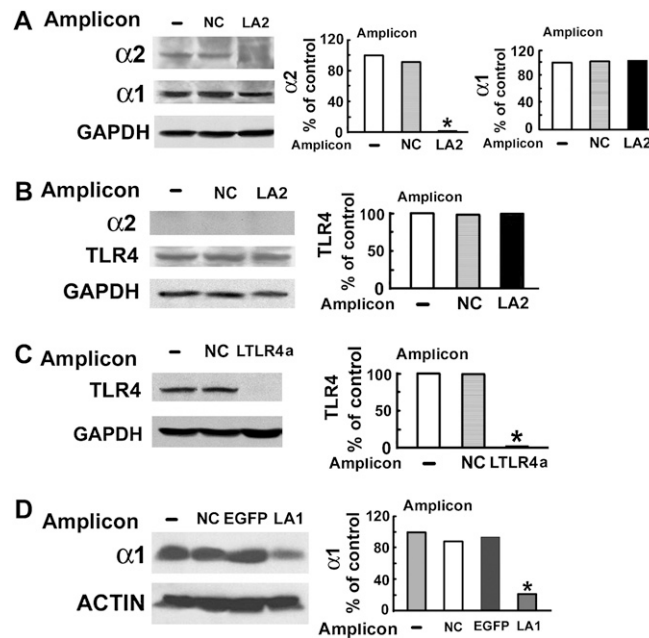
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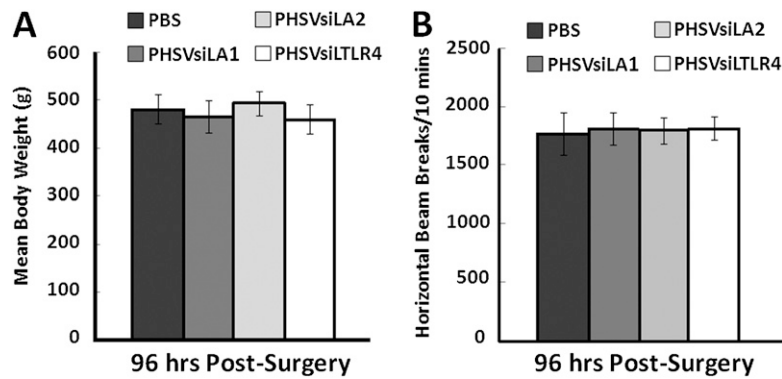




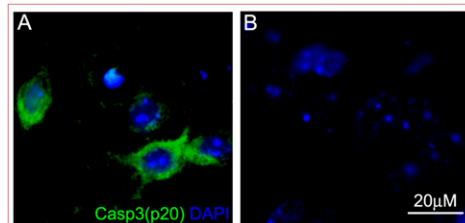
**Fig. S2.** Schematic representation of the siRNA plasmids. The HSV-1 amplicon vector pHSVsi contains three HSV-1 elements: an origin of DNA replication (OriS), the DNA packing/cleavage signal (Pac), and the IE4/5 promoter, which controls expression of the EGFP reporter gene. Sequences encoding siRNA with 19-nt homology (19nt) to the target sequences are synthesized as 60-mer sense and antisense oligonucleotide templates and inserted between the BglIII and HindIII sites downstream of the RNA polymerase  $\beta$ -dependent H1 promoter.



**Fig. S3.** Small interfering RNA (siRNA) amplicon vectors specifically inhibit expression of their cognate target genes. (A) RINm5F cells were mock transduced with PBS (–) or transduced with pHSVsiLA2 or pHSVsiNC and cell lysates collected 48 h later were immunoblotted with antibody to  $\alpha 2$ . The blots were stripped and sequentially reprobed with antibodies to  $\alpha 1$  followed by GAPDH used as loading control. The results were quantified by densitometric scanning and are expressed as the percent of control  $\pm$  SEM. The levels of  $\alpha 2$  were significantly decreased in cells transduced with pHSVsiLA2 compared with mock-transduced cells [ $F_{(2, 6)} = 386.34, P < 0.001$ ], but  $\alpha 1$  expression was not reduced [ $F_{(2, 6)} = 0.270, P > 0.05$ ]. pHSVsiNC did not alter gene expression. The Tukey post hoc test confirmed the effects of pHSVsiLA2 ( $*P < 0.01$ ) and pHSVsiNC ( $P > 0.05$ ) control treatments. (B) RAW264.7 cells that do not express  $\alpha 2$  but are positive for TLR4 were transduced with the pHSVsiLA2 or pHSVsiNC vectors. Protein extracts collected 48 h later were immunoblotted with antibodies to  $\alpha 2$  or TLR4 and the results are expressed as in A. pHSVsiLA2 did not inhibit TLR4 expression in these cells and pHSVsiNC was negative [ $F_{(2, 6)} = 2.28, P > 0.183$ ]. (C) RAW264.7 cells were transduced with pHSVsiTLR4a or pHSVsiNC and protein extracts collected 48 h later were immunoblotted with TLR4 antibody. The results are expressed as in A. TLR4 expression was markedly inhibited by pHSVsiTLR4a, but not pHSVsiNC, resulting in a highly significant treatment effect [ $F_{(2, 6)} = 129.134, P < 0.001$ ]. The Tukey test confirmed the effects of the pHSVsiTLR4a treatment ( $*P < 0.01$ ). (D) WS-1 cells were untreated (–) or transduced with pHSVsiNC, pHSVsiEGFP or pHSVsiLA1. Cell lysates collected at 48 h after transduction were immunoblotted with  $\alpha 1$ -specific antibody. The blots were stripped and reprobed with actin antibody as a gel loading control. The results are expressed as in A. pHSVsiLA1 reduced  $\alpha 1$  expression compared with untreated cells (–), or cells treated with the control amplicons pHSVsiNC or pHSVsiEGFP, resulting in a significant treatment effect [ $F_{(3, 8)} = 192.20, P < 0.001$ ], which was followed by the Tukey test ( $*P < 0.01$ ).



**Fig. 54.** Amplicon vectors do not alter body weight or locomotor measurements in the open-field in P rats. (A) Body weights and locomotor measures for animals given PBS, pHSVsiLA1, pHSVsiLA2, or pHSVsiTLR4 ( $n = 8$  each; total = 32) at 96 h postsurgery (4 d). No significant differences were observed between groups for body weight [ $F_{(3, 28)} = 0.252$ ,  $P > 0.05$ ]. (B) Locomotor activity (i.e., horizontal activity over a 10-min period) was also similar for animals given PBS, pHSVsiLA1, pHSVsiLA2, or pHSVsiTLR4 ( $n = 8$  each; total = 32) at 96 h postsurgery (4 d) [ $F_{(3, 28)} = 0.0278$ ,  $P > 0.994$ ]. No significant differences between groups were observed.



**Fig. 55.** Amplicons fail to induce apoptosis after intrastriatal injection. Adult male 129S6 SV/EV mice (Taconic) were anesthetized by intraperitoneal injection of 1.25% Avertin (0.025 mL/g body weight) and given intrastriatal injections by stereotax, as previously described (17). A cannula (33-gauge) containing the solution (0.5  $\mu$ L) to be delivered was lowered through the dura mater 3.0 mm via a small hole drilled 1.0 mm anterior and 2.5 mm lateral to the bregma. A microinjection pump (CMA/100; Carnegie Medicine) regulated the solution flow rate (0.1  $\mu$ L/min) over a period of 5 min; the cannula remained in place for an additional 3 min to permit diffusion. Afterward, the cannula was removed and the incision was closed. (A) Striatal-containing tissues from mice injected with HSV-1 were obtained at the time of death (5–11 d postinfection) and stained with FITC-labeled antibody to caspase-3p20 (activated caspase) using DAPI staining as control. (B) Mice injected with PBS or with pHSVsiLA2, pHSVsiLA1, pHSVsiTLR4a, or pHSVsiNC amplicon vectors lived for the 100 d of observation without displaying any untoward symptom. At this time striatal-containing tissue sections were stained with FITC-labeled antibody to caspase-3p20. As shown for pHSVsiLA2-infected mice, there was virtually no caspase-3p20 staining in any of the striatal cultures from animals in all groups.





