Supporting Information

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SI Results

EtOH Consumption Was Not Different Among Groups Before Deprivation. To assess whether rats were performing asymptotically before the termination of daily, operant self-administration sessions, data from the last 10 days of operant ethanol self-administration were analyzed by repeated-measures ANOVA over time. A comparison between 24 h depr and 3 wk depr revealed no difference in active lever presses [deprivation status: $F(1, 35) = 0.10$, $P = 0.76$; days: $F(9, 35) = 0.88$, $P = 0.54$; interaction: $F(9, 315) = 0.98$, $P = 0.46$ with $P > 0.45$ at any time point] or inactive lever presses [deprivation status: $F(1, 35) = 0.82$, $P = 0.37$; days: $F(9, 35) = 1.75$, $P = 0.08$; interaction: $F(9, 315) = 1.46$, $P = 0.16$ with $P > 0.11$ at any time point]. Moreover, no difference was found in g/kg consumption over this same time period [deprivation status: $F(1, 35) = 0.32$, $P = 0.58$; days: $F(9, 35) = 1.47$, $P = 0.16$; interaction: $F(9, 315) = 1.14$, $P = 0.33$ with $P > 0.21$ at any time point].

Enhanced Breakpoint After 3 Weeks of Deprivation Was Not Related to Days of Self-Administration Training. Because the greater number of training days in the 24 h depr group (10 wk training) relative to the 3 wk depr group (7 wk training) may have skewed the data, we performed additional studies employing 15 rats that were ≈3 wk older at time of arrival so that age at testing as well as days of training would be equivalent to the 3 wk depr group (7 wk training). When these older rats were tested for the motivation to seek ethanol after 24 h depr, no differences were found [breakpoint 10 wk training: 12.9 ± 1.9; breakpoint 7 wk training: 15.3 ± 2.5 with $F(1, 31) = 0.64$, $P = 0.43$]. In addition, no differences were found in active lever presses [normal: 73.44 ± 10.23; older: 97.7 ± 20.4 with $F(1, 31) = 1.25$, $P = 0.27$] or in inactive lever presses [normal: 8.3 ± 2.0; older: 7.1 ± 1.8 with $F(1, 31) = 0.18$, $P = 0.67$].

Moreover, a comparison of 24 h depr rats with 10 wk (original design) and 7 wk (new experiments) of self-administration training revealed a similar asymptote in active lever presses [onset age: $F(1, 26) = 0.90$, $P = 0.35$; days: $F(9, 26) = 0.85$, $P = 0.57$; interaction: $F(9, 234) = 1.36$, $P = 0.21$ with $P > 0.13$ at any time point] and inactive lever presses [onset age: $F(1, 26) = 0.95$, $P = 0.34$; days: $F(9, 26) = 0.50$, $P = 0.87$; interaction: $F(9, 234) = 1.10$, $P = 0.36$ with $P > 0.12$ at any time point]. Accordingly, no differences were observed in g/kg consumption [onset age: $F(1, 26) = 1.73$ $P = 0.20$; days: $F(9, 26) = 0.82$, $P = 0.60$; interaction: $F(9, 234) = 1.57$, $P = 0.13$ with $P > 0.18$ at any time point].

Blood Alcohol Was Correlated with Breakpoint. Analysis of data from 3 wk depr rats after breakpoint determination revealed a significant correlation of blood ethanol concentration (BEC) to the number of ethanol reinforcer deliveries ($R^2 = 0.59$, $P < 0.05$), where the BEC attained was 3.97 ± 0.93 mg% (max: 11.50 mg% to min: 0.81 mg%; $n = 14$). Analysis of 24 h depr data also revealed a positive correlation of BEC with ethanol reinforcer deliveries ($R^2 = 0.33$, $P < 0.05$) where the BEC attained was 2.07 ± 0.15 mg% (max: 2.94 mg% max; min: 1.22 mg%; $n = 14$). Furthermore, 3 wk depr rats consumed more ethanol than 24 h depr rats [$F(1, 26) = 4.08$, $P < 0.05$].

BEC measurements at the end of a FR3 session were 4.05 ± 0.29 mg% ($n = 30$). These data were higher than the BEC from 24 h depr rats tested on the progressive ratio schedule during an ≈1-h session [$F(1, 42) = 20.07$, $P < 0.01$]. Interestingly, however, these data were not different from the BEC in 3 wk depr rats on the progressive ratio test day [$F(1, 42) = 0.01$, $P = 0.92$].

Of further interest is that our results in Fig. 2 *E* and *F* suggest that the motivation expressed to seek ethanol in 3 wk depr rats was independent of ethanol consumption, because the breakpoint attained when ethanol access was blocked by a Plexiglas barrier was not different from that attained when ethanol was available. Thus, the expression of the enhanced motivation for ethanol after 3 wk of abstinence did not depend on receipt of the ethanol reinforcer, even though ethanol was consumed.

Breakpoint Was Correlated with EtOH Self-Administration History. Our results in Fig. 2 *E* and *F* suggest that the motivation expressed to seek ethanol after 3 wk of deprivation did not require ethanol consumption. In contrast, the motivation to seek ethanol was related to the level of ethanol self-administration before abstinence. Accordingly, analysis of breakpoint data in relation to prior self-administration history revealed that breakpoint after either 24 h or 3 wk of deprivation was correlated with active lever pressing (3 wk depr: $R^2 = 0.28$, $P < 0.05$; 24 h depr: $R^2 = 0.47$, $P < 0.01$) as well as to g/kg consumed (3 wk depr: $R^2 = 0.56$, $P < 0.01$; 24 h depr: $R^2 = 0.52$, $P < 0.01$) during the last 10 days of operant self-administration.

AGS3 Expression Was Correlated with EtOH Breakpoint After Deprivation. To determine whether AGS3 protein expression levels correlate with the altered motivation to lever-press for ethanol, we examined the relative immunoreactivity values obtained from immunoblotting of gross tissue dissections in relation to breakpoint. These data suggest that a positive correlation between AGS3 expression and breakpoint may exist. In 3 wk depr rats, there was a nearly significant positive correlation between AGS3 expression and breakpoint, with an $R^2 = 0.48$ ($P = 0.056$), whereas in 24 h depr rats there was no relationship between breakpoint and AGS3 expression ($R^2 = 0.09$, $P = 0.47$).

To further examine the relationship between motivation to seek ethanol and AGS3 expression, an additional group was included where the motivation to press for ethanol was assayed after 3 wk of deprivation, and then once a week afterward for a total of 6 wk. This group was included because the alcohol deprivation effect has been shown to decay after multiple re-exposures to ethanol (1). In agreement, the breakpoint in this group after 3 wk of deprivation was significantly elevated relative to 24 h depr rats [18.19 ± 1.70, $F(1, 32) = 4.37$, $P < 0.05$, $n = 16$] but decayed across subsequent weeks of testing (breakpoint wk 4: 16.19 ± 1.73; breakpoint wk 5: 14.53 ± 1.15; breakpoint wk 6: 11.31 ± 1.27). Thus, the breakpoint of the final test was different from that expressed by 3 wk depr rats [$F(1, 32) = 17.83$, $P < 0.01$], but was not different from that expressed by 24 h depr rats [$F(1, 32) = 0.47$, $P = 0.50$]. Interestingly, the breakpoint of the final test was negatively correlated to AGS3 expression ($R^2 = 0.05$, $P < 0.01$). This suggests that the reduction in motivation to seek ethanol across repeated testing (1) was accompanied by a reduction in AGS3 expression, with higher AGS3 expression in rats showing a smaller decay in breakpoint by the final test. These results further support a relationship between NAcore AGS3 expression and ethanol-seeking motivation.

In contrast, in rats with an extended 6-wk abstinence period, AGS3 expression returned to baseline AGS3 expression (Table S1), even though breakpoint after 6 wk of abstinence was enhanced relative to 24 h depr rats [6 wk of abstinence: 21.6 ± 2.8, $F(1, 34) = 6.72$, $P < 0.05$] and was not different from rats deprived for 3 wk [$F(1, 34) = 0.24$, $P = 0.63$]. Furthermore, no correlation was found between AGS3 expression and breakpoint

after 6 wk of deprivation ($R^2 = 0.05$, $P = 0.29$). Thus, in animals that have undergone more prolonged abstinence, elevated NAcore AGS3 is no longer necessary for the enhanced motivation for ethanol, suggesting that elevated AGS3 may facilitate the development of more persistent neuroadaptations that support enhanced ethanol seeking during more prolonged periods of abstinence.

These data suggest that AGS3 protein expression was correlated with the rise and fall of motivation to seek ethanol during the initial weeks of protracted abstinence. In contrast, in animals undergoing a more prolonged abstinence, a mechanism other than elevated NAcore AGS3 likely underlies the enhanced motivation to seek ethanol at this time point. In addition, our data suggest that weekly breakpoint testing removed rats from protracted deprivation, such that their motivation for ethanol was once again related to NAcore AGS3 expression. These data also agree with a previous report (2) in which knocking down AGS3 in the prefrontal cortex normalized several cocaine-modulated phenomena after 3 wk of abstinence but that AGS3 expression in the prefrontal cortex declined during more extended cocaine abstinence even though reliable reinstatement of cocaine-seeking behavior can be obtained (3). These results suggest that elevated AGS3 expression is critical for the aberrantly enhanced ethanol seeking during the initial weeks of protracted abstinence, and that neuroadaptations other than increased AGS3 support drug seeking after more prolonged periods of abstinence.

AGS3 Knockdown Did Not Affect Responding for Sucrose. Additional control experiments were performed that suggested no effect of AGS3 knockdown on responding for sucrose. In particular, AGS3 was knocked down in a group of sucrose-self-administering rats tested for breakpoint after 24 h depr ($n = 5$ per treatment). Breakpoint attained was as follows: AS, 37.8 ± 2.2; SC, 42 ± 2 [with $F(1, 8) = 2.00$, $P = 0.20$]. Analysis of active lever responding yielded $P = 0.91$ and inactive lever responding $P = 0.40$. When combined with 3 wk depr data, analysis of breakpoint suggested no effect of knockdown [$F(1, 40) = 1.85$, $P = 0.18$], deprivation [$F(1, 40) = 1.39$, $P = 0.25$], or interaction [$F(1, 40) = 0.10$, $P = 0.76$].

Motivation to seek sucrose was also assessed after 24 h depr in an additional group of nine rats that did not undergo surgery, yielding a breakpoint of 39.4 ± 3.74. Analysis of these data compared with those obtained under AGS3 knockdown after 3 wk depr suggested no effect of knockdown on breakpoint [$F(1, 24) = 1.63$, $P = 0.21$], active [$F(1, 24) = 2.75$, $P = 0.11$], or inactive [$F(1, 24) = 1.96$, $P = 0.17$] lever responding.

These data also concur with our observations that AGS3 knockdown did not reduce ethanol self-administration in animals where the breakpoint was measured after only 24 h of deprivation (Fig. 3 *A* and *B*). This is in stark contrast with the significant reduction in breakpoint by AGS3 knockdown in animals after 3 wk abstinence. These results strongly suggest that AGS3 knockdown only altered ethanol-related behavior under conditions where AGS3 levels were elevated.

In addition, these data, coupled with previous work on AGS3 expression changes in relation to cocaine reinstatement (2), suggest an interesting situation where AGS3 may serve as a gatekeeper, so that when AGS3 expression is above threshold, addiction-associated pathology ensues; however, when AGS3 expression is below threshold, addiction-associated pathology appears normalized, and reducing AGS3, which is already below threshold, does not influence nonpathological drug seeking. In other words, we hypothesize that the ability of AGS3 to pathologically facilitate drug seeking occurs when AGS3 levels increase above some threshold, and that decreasing AGS3 to or below that threshold leads only to normal self-administration.

Inactive lever responding. Analysis of inactive lever responding during knockdown, regardless of whether rats were seeking EtOH or sucrose, suggested no main effect or interaction [knockdown: $F(1, 104) = 0.00$, $P = 0.96$; deprivation: $F(1, 104) = 2.27$, $P = 0.13$; knockdown × deprivation: $F(1, 104) = 2.65$, $P = 0.11$; knockdown × deprivation × drink: $F(1, 104) = 0.15$, $P = 0.70$]. Individual analysis on means continued to suggest no effect during 3 wk of deprivation [AS-AGS3: 5.9 ± 1.6; SC-AGS3: 11.2 ± 2.4; $F(1, 26) = 3.30$, $P = 0.08$], after 24 h of deprivation [AS-AGS3: 13.5 ± 2.7; SC-AGS3: 10.8 ± 2.6—where $F(1, 39) = 0.49$, $P = 0.49$], or during 3 wk of deprivation from sucrose [AS-AGS3: 24.5 ± 4.6, $n = 17$; SC-AGS3: 30.8 ± 5.7, $n = 17$—with $F(1, 32) = 0.74$, $P = 0.40$].

Additional analyses under G$\beta\gamma$ sequestration also suggest no effect on inactive lever responding [βARK: 23.0 ± 4.9, $n = 8$, GFP: 18.5 ± 5.7, $n = 6$—with $F(1, 12) = 0.36$, $P = 0.56$].

SI Methods

Ethanol Self-Administration Training. Rats received ad libitum access to home-cage food during all procedures; water access is described below. Lights were on a 12-h cycle with lights on at 700 hours. All experimentation occurred between 1000 and 1700 hours except during overnight training sessions, as noted below. All rats were trained in standard operant chambers with retractable levers (Coulbourn Instruments), using a consumption-based paradigm, where access to the cup containing the reinforcer depended on active licking of the cup (see below for details).

After acclimation for 1 wk with ad libitum access to food and water, home-cage water was replaced with 10% EtOH for 5 days. On the evening of the sixth day, rats were placed in the operant chamber (with ad libitum access to food) for two to three overnight, 15-h, fixed ratio 1 (FR1) sessions for 10% sucrose on a consumption-based paradigm. After completion of the fixed ratio schedule of reinforcement, a 100-μl dipper cup containing the reinforcer was elevated, a stimulus light above the active lever was illuminated, a tone was activated, and the dipper cup was illuminated; all for 4 sec. If the cup was not actively licked during the first 2 sec, the cup fell and this event was recorded as a null response and not included in g/kg calculations. For the cup to rise again, the rat had to complete another fixed ratio schedule.

Water was available in the home cage for 1 h after the first overnight session and 3 h after the second (and/or third). After the last overnight session, rats advanced to a 45-min, FR1 session for 10% sucrose the next day followed by 6 h of home-cage access to water. The next day, rats were placed on a fixed ratio 3 schedule of reinforcement (FR3) for 10% sucrose in a 30-min session and afterward received ad libitum access to water in the home cage. Ad libitum access to both food and water were maintained from this point forward. During the subsequent days, training proceeded through a modified sucrose fade (4). The fade began at 10% EtOH in 10% sucrose, followed by 10% EtOH in 5% sucrose, then 10% EtOH in 3% sucrose, then 10% EtOH in 1.5% sucrose; then the rat was switched to 10% EtOH without sucrose. Rats remained at each ethanol/sucrose level for one to three sessions (one session per day), and each session was a 30-min FR3. Rats continued to respond on FR3 for 10% EtOH in 30-min sessions for 45–50 contiguous days. The first day of responding for 10% EtOH/0% sugar was considered day 1 of ethanol self-administration. Again, only reinforcers, and not null responses, were included in g/kg calculations, and only rats that consumed between 0.25 and 1.2 g/kg of EtOH per 30-min session per day (averaging ≈0.45 g/kg) during the last 10 days of training were used for experimentation. Approximately 3% of the rats were excluded for low intake or failure to acquire the operant response.

Rats intended for sucrose-seeking experiments were deprived

of water in the home cage for 2 days after acclimating to the facility for 1 wk. On the evening of the second day, rats were placed into the operant chamber with ad libitum access to food for one of two to three overnight, 15-h, FR1 sessions for 5% sucrose on the consumption-based paradigm described above. Water was available in the home cage for 1 h after the first session and 3 h after the second and/or third. After the last session, rats advanced to a 45-min, FR1 session for 5% sucrose the next day followed by 6 h home cage access to water. The next day, rats responded on a FR3 for 5% sucrose in a 30-min session and received ad libitum access to water in the home cage. Ad libitum access to both food and water were maintained from this point forward, and total days of self-administration training were equal to the EtOH group, with day 1 being the first day of FR3 responding. Approximately 1% of the rats did not acquire the operant response.

Surgery. Rats were anesthetized via isoflurane with O_2 as the carrier (to effect) and implanted with 26-gauge, chronically indwelling stainless steel guide cannulae (Small Parts) aimed 1 mm above the nucleus accumbens core at +1.2 mm AP, ±1.5 mm ML, and −6.0 mm DV from bregma according to the atlas of Paxinos and Watson (5) and obturated with 33-gauge stainless steel (Small Parts), as described in ref. 6, or 28-gauge, bilateral side arm cannulae that were lowered an additional millimeter (Plastics One) when oligonucleotide was to be delivered. Oligonucleotide infusion necessitated that two, 2-week osmotic minipumps be s.c. implanted (Model 2002; Alzet/Durect), as described in ref. 2. Cannulae implantation for virus injection generally occurred during the first week of EtOH abstinence. Surgery to implant osmotic minipumps occurred 2 wk into abstinence so that oligonucleotide infusion continued through test day.

Gpsm1/AGS3 Antisense and βARKct Viral-Mediated Delivery. Cannula implantation was performed as described in ref. 2 after 7 days of abstinence. Virus injections occurred 7 days after surgery. Adenovirus serotype 5 carrying the C-terminal fragment of β-adrenergic receptor kinase (βARKct) minigene, GFP, Gpsm1/AGS3 antisense, or Gpsm1/AGS3 scrambled under the cytomegalovirus promoter was constructed, amplified, and purified, as described in ref. 7. Virus was injected via a 33-gauge stainless steel needle (Small Parts) into the NAcore at 10^6 pfu/μl in a volume of 1 μl at a rate of 0.1 μl/min. An additional 10 min elapsed before microinjector removal and 33-gauge stainless steel obdurator replacement (Small Parts). Behavioral and immunochemical testing occurred 7 days after injection. Viral-mediated antisense efficacy was determined in 3-wk-EtOH-deprived rats by immunoblotting 1 wk after injection of the antisense construct into one hemisphere compared with a contralateral injection of the control construct.

Giα Antisense Design and Delivery. BLASTn searches (8) were conducted, as described in ref. 2, to design DNA antisense and scrambled constructs that hybridize to $Gi\alpha_{1/3}$ mRNA specifically or to no known gene, respectively. Constructs were synthesized with five phosphorothioate bonds ("*") at each end and purified by reversed-phase HPLC (Integrated DNA Technologies). Scrambled, 5′-C*A*G*A*C*GCCCGCCGGACCCGAGAT-GATGG*C*C*T*T*A-3′; $Gi\alpha_1$, 5′-G*C*T*G*T*CCTTCCA-CAGTCTCTTTATGACG*C*C*G*G*C-3′; $Gi\alpha_3$, 5′-G*C*-C*A*T*CTCGCCATAAACGTTTAATCACG*C*C*T*G*C-3′. Constructs were delivered individually by osmotic minipumps (Alzet) at 42 μM in saline through cannula directed into the NAcore at 500 pmol/24 h. Surgery occurred 10–14 days after onset of EtOH abstinence, allowing ≈1 wk of continuous infusion and recovery before test. Knockdown of $Gi\alpha_1$ did not affect expression of $Gi\alpha_{2/3}$ [$Gi\alpha_2$: 98.3 ± 6.5%, $n = 6$; $Gi\alpha_3$: 1.14 ± 21.5%, $n = 6$; $F(1, 10) = 0.48$, $P = 0.51$]. Similarly, knockdown of $Gi\alpha_3$ did not affect expression of $Gi\alpha_{1/2}$ [$Gi\alpha_1$: 92.9 ± 15.6%, $n = 6$; $Gi\alpha_2$: 1.10 ± 21.5%, $n = 6$; $F(1, 10) = 0.57$, $P = 0.48$].

Breakpoint Determination. Responding was initiated by presentation of a compound cue that consisted of lever extension, stimulus light illumination, and tone sounding, as well as illumination of a raised dipper cup filled with either 10% EtOH or 5% sucrose. In addition, rats were presented with an EtOH odor cue generated by sprinkling ≈15 ml of ≈87% EtOH beneath the previously EtOH-paired lever, and rats were exposed to the ethanol odor cue for 2 min before presentation of the compound cue; the bedding of sucrose and "no-cue" EtOH rats was sprinkled with water. Rats were free to choose to drink from the cup, press a lever, or do nothing. If the rat chose to do nothing, a 20-sec timeout period occurred and the rat was re-cued for up to 20 iterations. One to two re-cues were sufficient to elicit a response, which was split by ≈50% lick first and 50% press first. If rats licked first, the progressive ratio schedule of reinforcement of 1, 1, 2, 2, 3, 4, 5, 6, 7, 9, 10, 12, 13, 15, 17, 20, 22, 25, 28, 32, 36, 40, 45, 50, etc. ensued. If rats pressed first, the progressive ratio was 1, 2, 2, 3, 4, 5, 6, 7, 9, 10, 12, 13, 15, 17, 20, 22, 25, 28, 32, 36, 40, 45, 50, etc. (see also Fig. S1).

Blood Alcohol Levels. Blood alcohol concentrations were determined by adapted gas chromatographic (GC) procedures (9) as described previously for brain ethanol (10) with a 0.01 mM limit of detection. Briefly, rats were lightly anesthetized with isoflurane (to effect), and ≈300–500 μl of blood was collected from the lateral, rostral tail vein by venous puncture. A warming lamp was not used. Blood was vigorously mixed with inversion and centrifuged at 5,000 × *g*, 4°C for 20 min. Serum was decanted, vortexed, and 10 μl serum was sealed in a GC autosampler vial (National Scientific) with 10 μl of 0.05% *n*-propanol as an external pippeting standard. Samples, in triplicate, were heated to 65°C for 20 min, agitated for 30 sec, and allowed to settle for 1 min before pressurizing for headspace extraction into a 2-ml, depolarized loop (Tekmar Control Systems). Samples were immediately passed through a 220° deactivated, glass-lined inlet (Hewlett–Packard) and subjected to gas chromatography (He, 5 kPa) on a megabore 30-m, 1-μm film INNOWAX column (Agilent Technologies) at a 45°C isotherm and quantified by flame ionization at 310°C (HP5890; Hewlett–Packard). The column was purged after each sample by holding at 210°C for 1.5 min before cooling to 45°C over 5 min. The alcohol area under the curve (AUC) was divided by the external *n*-propanol standard AUC and compared with known ethanol standards from 300 to 0.003 mM (run in duplicate, R^2 typically ≈1.00) that were freshly prepared for every run before mg% alcohol concentration calculation (11).

Rotarod. Motoric capacity was assessed in EtOH-naïve rats, 3-wk-EtOH-deprived rats, and 3-wk-EtOH-deprived rats previously transfected with the βARK minigene. Rats were habituated to a stationary, textured rotarod (7 cm in diameter, lane width of 9 cm) located one foot off the table surface until they could remain on the rod with no rotation for 2 min, which generally took three to five trials separated by 5 min. Next, rats were allowed to navigate the rod rotating at 2.5 rpm for 10 trials. The next day, as illustrated in Fig. S3, rats navigated the rod at 5 rpm for five trials and then at 10 rpm for an additional five trials. Rats were not allowed to navigate the rotarod for >300 sec in any trial, and all rats experienced at least a 10-min rest between trials to control for exhaustion, except during initial training on the stationary rod.

Open Field Locomotion. Exploration of a novel, open field was assessed in the same rats the day after rotarod assessment. The open field consisted of a 43 × 43 cm Plexiglas cube equipped with 16 infrared photobeam pairs to measure *x–y* ambulatory movements at a 50-msec scanning rate and were individually encased in sound attenuating cabinets (Med Associates). Rats were naïve to the apparatus and placed in the center of the open field at the start of experimentation. Distance traveled (determined by consecutive breaking of adjacent photobeams, reported in centimeters) was measured by using Activity Monitor software (Med Associates). Sixty minutes later, saline (1 ml/kg, i.p.) was given to all subjects immediately before placing them back into the center of the open field for an additional 30 min. Exclusion of a habituation period to the open field environment allowed for the assessment of novelty-induced exploration and avoided potential floor effects.

Histology. Rats not slated for immunochemical experiments were anesthetized with pentobarbital (100 mg/kg, i.p.) and transcardially perfused with cold PBS, followed by 4% freshly prepared paraformaldehyde in PBS; the brain was then removed and postfixed in 2% paraformaldehyde at 4°C. Coronal sections (100 μm thick) of the cannula tract were obtained with a vibratome (Leica) and stained with cresyl violet. Photomicrographs were obtained with a Nikon Eclipse E600 microscope in brightfield, using a Spot CCD camera (Diagnostic Instruments).

Immunoblotting. Gpsm1/AGS3 blotting was conducted essentially as described in ref. 2, except that, because of the availability and increased linearity of infrared detection, brain extracts were transferred to 0.22-μm nitrocellulose (Amersham Biosciences) and even transfer verified by using Ponceau S (Sigma–Aldrich). After de-staining, membranes were incubated with rabbit Pep22 antisera against Gpsm1/AGS3 (Chemicon/Millipore) at 1:2,000, 4°C, overnight in 4% milk and labeled with donkey × rabbit IR800 secondary (Rockland Immunochemicals) at 1:10,000 at room temperature (RT) for 90 min. To detect Gi$\alpha_{1/3}$ expression, tissues were dissected and homogenized as described in ref. 2. For Giα_1, 5-μg homogenized samples were prepared in Laemmli sample buffer, loaded onto a freshly cast 10% SDS/PAGE gel, and transferred, as above. Blots were probed overnight with monoclonal antisera at 1:40,000 (Chemicon/Millipore) in 4% milk at 4°C, and then labeled with Alexa Fluor 680 goat × mouse (Molecular Probes/Invitrogen) at 1:10,000 for 90 min in 4% milk at RT. For Giα_3, 15 μg of homogenized samples were electrophoresed and transferred, as above, and blots were probed with chicken antisera (Chemicon/Millipore) at 1:2,000 in 4% milk at 4°C overnight before labeling with IR700DX goat × chicken (Rockland Immunochemicals) at 1:7,000 in 4% milk at RT for 90 min. Loading concentration and antisera dilution was determined to be within the linear range of detection (Licor Odyssey Biosciences). Blots were quantified by integrated intensity, which is a measure of pixel density that is independent of image resolution and of the size of the bounding box drawn to define lanes and bands.

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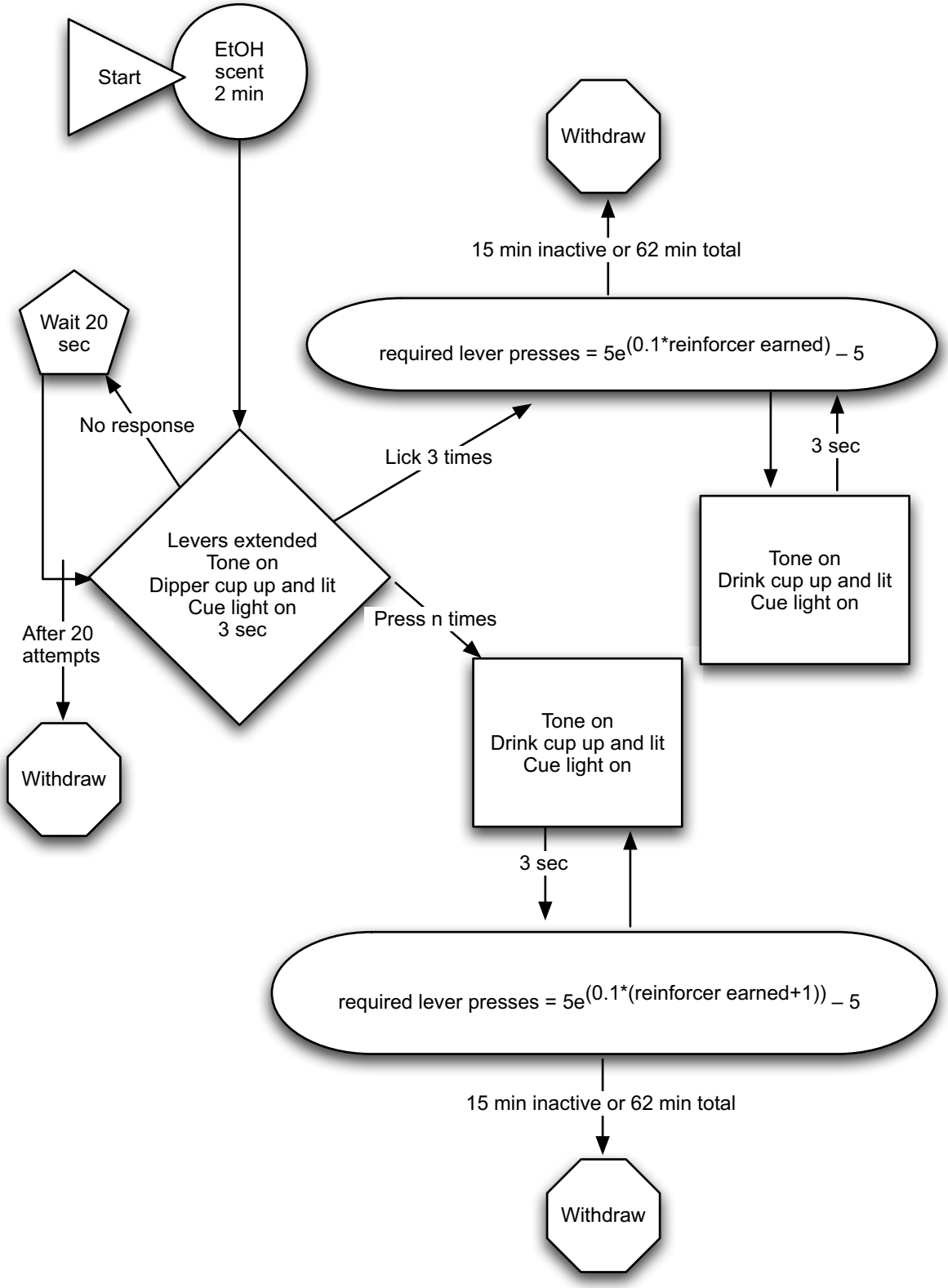

Fig. S1. Flowchart for PR experiments. Shown is a schematic of experimental flow during the PR experiments.

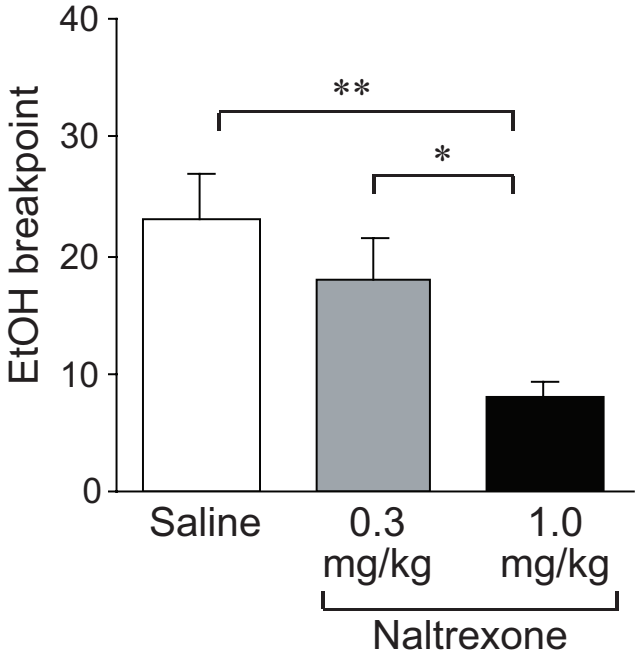

Fig. S2. Naltrexone dose-dependently reduced EtOH seeking. Naltrexone or saline vehicle at 1 ml/kg was given s.c. in the home cage 13 min before placing rats in the operant chamber for a progressive ratio session. Naltrexone reduced the willingness to work for EtOH on a progressive ratio [$F(2, 18) = 4.91$, $P < 0.05$; saline: $n = 7$; 0.3 mg/kg: $n = 8$; 1 mg/kg: $n = 6$]. Data represent mean ± SEM. *, $P < 0.05$, using ANOVA with Scheffé post hoc comparisons.

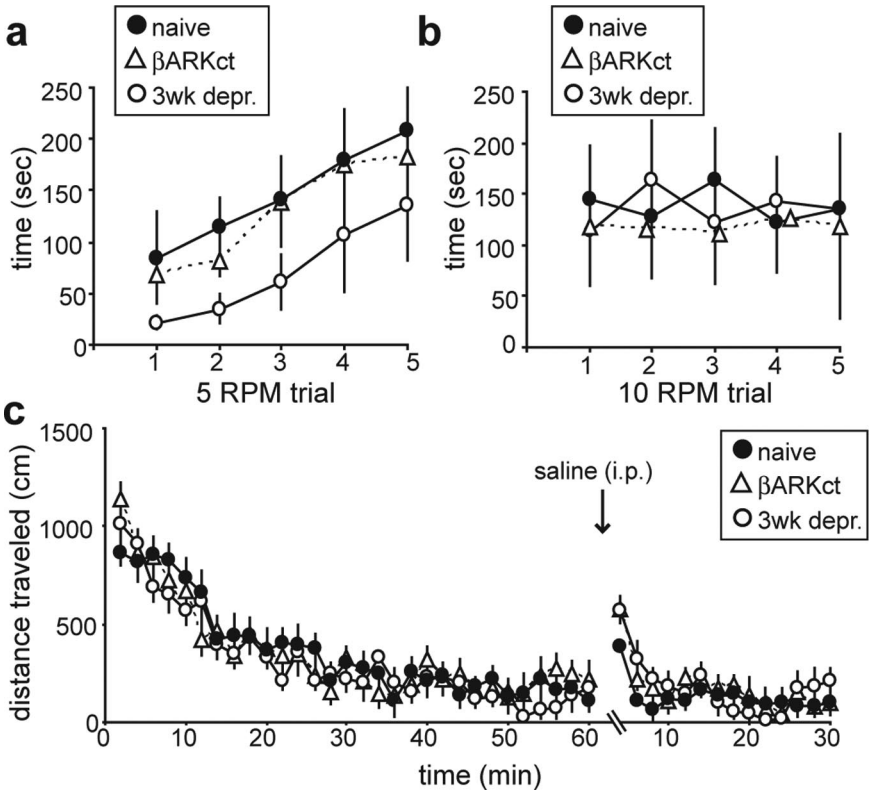

Fig. S3. βARKct overexpression did not affect motoric capacity. Because a trend toward decreased overall rate of responding was observed under βARKct overexpression [ctβARK: 0.038 ± 0.007 presses per sec; GFP: 0.058 ± 0.007 presses per sec; $F(1, 12) = 3.65$, $P = 0.08$] that was not apparent under AGS3 or $Gi\alpha_{1/3}$ knockdown [AS-AGS3: 0.054 ± 0.014 presses per sec; SC-AGS3: 0.059 ± 0.009 presses per sec; $F(1, 26) = 0.09$, $P = 0.77$; $Gi\alpha_1$-AS: 0.057 ± 0.013 presses per sec; $Gi\alpha_3$-AS: 0.041 ± 0.008 presses per sec; Giα-SC: 0.038 ± 0.008 presses per sec; $F(2, 23) = 1.03$, $P = 0.37$], two motoric measures were assessed after βARKct transfection in 3 wk depr rats and in EtOH-naïve rats. (*a* and *b*) Rats trained the previous day to navigate a rotarod at 2.5 rpm were tested at 5 rpm (*a*) and 10 rpm (*b*), with five consecutive trials at each speed and a 10-min rest between the 5-rpm and 10-rpm trials. Trials were capped at 300 sec. (*a*) Rats learned to navigate the rotarod at 5 rpm [repeated-measures ANOVA: $F(4, 4) = 8.09$, $P < 0.01$], but there was no effect of βARKct overexpression in 3 wk depr rats [$F(2, 13) = 1.84$, $P = 0.20$] or interaction [treatment × trial: $F(8, 52) = 0.08$, $P = 1.00$]. (*b*) At 10 rpm, no effect of trial [$F(2, 13) = 0.06$, $P = 0.95$], treatment [$F(4, 4) = 0.15$, $P = 0.96$], or interaction [$F(8, 52) = 1.40$, $P = 0.22$] was observed, indicating that rats generally navigated the rotarod the same regardless of βARKct overexpression or EtOH deprivation. (*c*) The next day, locomotion in a novel, open field was assessed both before and after mild hand restraint stress followed by an injection of saline (1 ml/kg, i.p.). No differences were observed in distance traveled before injection [repeated-measures ANOVA; treatment: $F(2, 13) = 0.33$, $P = 0.73$; treatment × habituation time $F(118, 767) = 0.94$, $P = 0.66$] or after injection [treatment: $F(2, 13) = 1.71$, $P = 0.22$; treatment × response time $F(58, 377) = 1.21$, $P = 0.15$]. Data are expressed as mean motoric activity ± SEM and analyzed by two-way repeated-measures ANOVA. Depr., 3 wk of EtOH deprivation; naïve, intact; EtOH-naïve, age-matched controls; cm, centimeters.

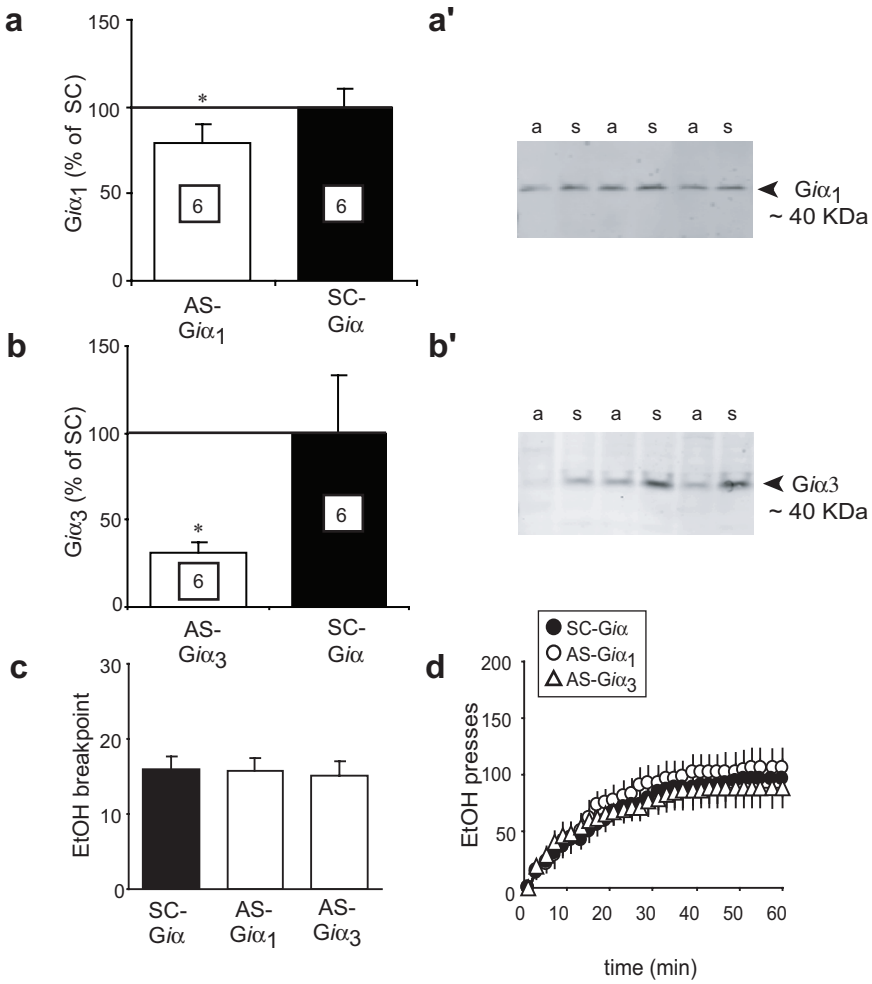

Fig. S4. Antisense directed against Gi$\alpha_{1/3}$ did not affect EtOH seeking. After ≈1 wk of infusion into the NAcore of 3-wk-EtOH-deprived rats of 504 pmol/24 h of a minimally phosphothioated antisense oligonucleotide directed against the initiation codon region of Giα_1 or Giα_3 or pan Gi$\alpha_{1/3}$ scrambled oligonucleotide, the region surrounding the cannulae tract was excised and subjected to immunoblotting for Giα_1 (*a*) or Giα_3 (*b*). Data represent mean ± SEM. Integrated intensity of one hemisphere that was infused with antisense compared to the contralateral side that was infused with a pan Giα scrambled construct ($n = 6$ for all treatments). Representative blots for Giα_1 (*a′*) or Giα_3 (*b′*). (*c*) Gi$\alpha_{1/3}$ knockdown in the NAcore did not reduce breakpoint in 3-wk-EtOH-deprived rats compared with rats infused with pan-Giα scrambled oligonucleotide designed to not bind any known gene (Giα_1 AS: $n = 7$; Giα_3 AS: $n = 10$; scrambled: $n = 9$). Data represent mean ± SEM. *, $P < 0.05$, using paired *t* test. a, antisense; s, scrambled.

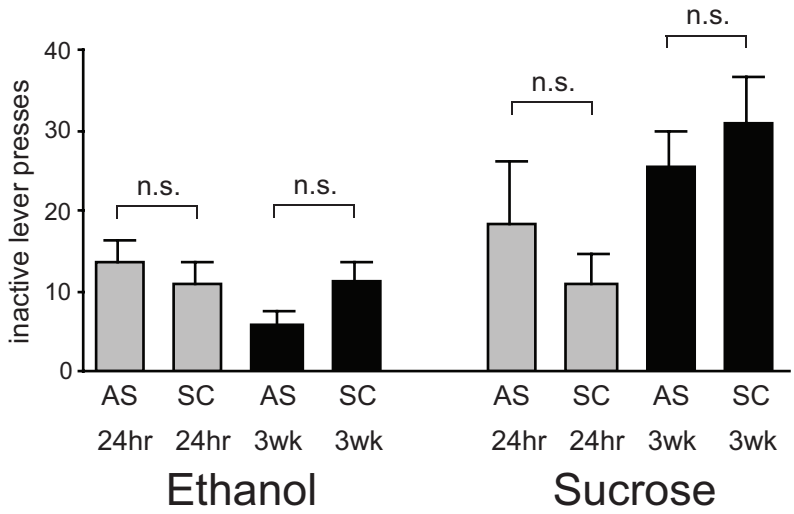

Fig. S5. Lack of AGS3 knockdown on inactive lever responding. Inactive lever responding is shown for experiments where AGS3 expression was knocked down. Inactive lever responding was assessed after either 24 h or 3 wk of deprivation from either EtOH or sucrose operant self-administration. No significant effects were found. Data represent mean ± SEM. AS, antisense; SC, scrambled; 24 h, 24 h of deprivation; 3 wk, 3 weeks of deprivation; n.s., not significant at $P < 0.05$, using a three-way ANOVA.

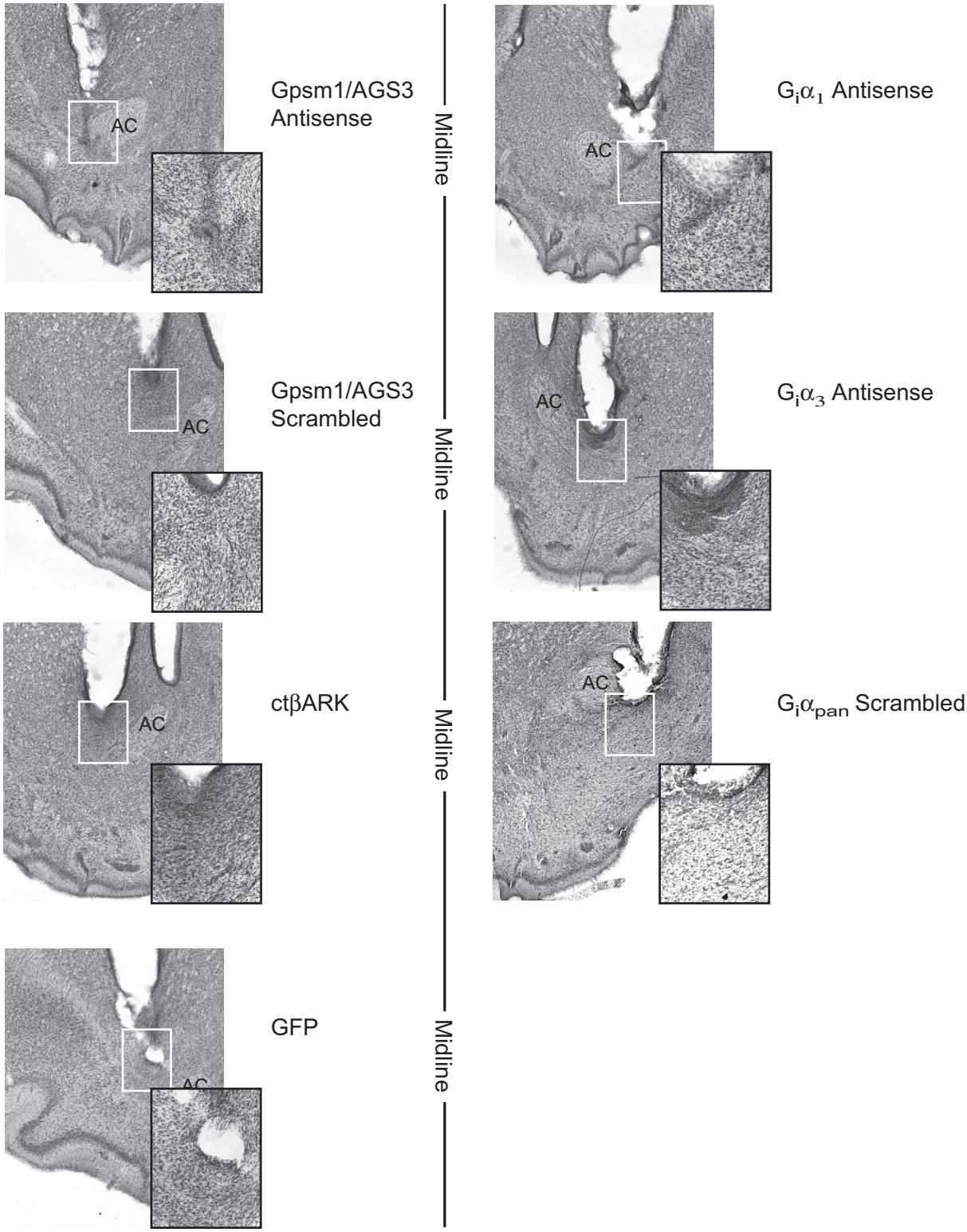

Fig. S6. Representative histology. Nissl staining of tissues at ×4 from experimental groups described above suggested that no overt toxicity occurred after adenoviral injection or oligonucleotide infusion. Midline is oriented toward the middle of the figure. AC, anterior commissure. Inset ×20.

Table S1. Forebrain AGS3 expression during EtOH abstinence

	3 wk abstinence			6 wk abstinence	
	Naïve O.D.	Deprived O.D.	Nondeprived O.D.	Naïve O.D.	Deprived O.D.
NAcore	100 ± 6.3 (11)	125.6 ± 9.6* (12)	105.4 ± 4 (13)	100 ± 8.4 (17)	92.7 ± 7.4 (18)
NAshell	100 ± 8.1 (11)	112.4 ± 8.2 (13)	85 ± 6.8 (10)	100 ± 7.8 (12)	91.8 ± 15 (15)
STR	100 ± 4.6 (11)	108.5 ± 4.9 (13)	97.3 ± 14.1 (13)	100 ± 11 (12)	89.4 ± 9.6 (16)
PFC	100 ± 7.2 (11)	94.6 ± 7.9 (14)	101.4 ± 14.8 (13)	100 ± 6.4 (12)	87 ± 5.8 (16)

Data represent percent expression of optical density values from naïve animals ± SEM (*n*). NAcore, nucleus accumbens core; NAshell, nucleus accumbens shell; STR, dorsal striatum; PFC, infralimbic and prelimbic prefrontal cortex. *, $P < 0.05$, comparing deprived to naïve and nondeprived animals using a one-way ANOVA with Scheffé post hoc comparisons.

Table S2. Statistical analysis summary of motivational assessments

	Comparison	*F*	*P*
	Breakpoint		
No virus	3 wk vs. 24 h depr	(1, 34) = 11.77	< 0.01
Barrier	3 wk depr barrier vs. 24 h	(1, 25) = 5.21	< 0.05
	3 wk depr vs. 3 wk barrier	(1, 25) = 0.264	= 0.61
No-cue	3 wk depr no-cue vs. 24 h	(1, 26) = 3.24	= 0.08
	3 wk depr no-cue vs. 3 wk barrier	(1, 16) = 13.09	< 0.01
AGS3 knockdown 3 wk depr			
EtOH	AS-AGS3 vs. SC-AGS3	(1, 26) = 8.48	< 0.01
	AS-AGS3 vs. no surgery	(1, 29) = 12.03	< 0.01
	AS-AGS3 vs. 24 h depr	(1, 29) = 0.11	= 0.74
	SC-AGS3 vs. 3 wk depr	(1, 31) = 0.15	= 0.71
Sucrose	AS-AGS3 vs. SC-AGS3	(1, 32) = 2.56	= 0.12
	AS-AGS3 vs. no virus 24 h depr	(1, 24) = 1.63	= 0.21
24 h depr			
EtOH	AS-AGS3 vs. no virus 24 h depr	(1, 27) = 0.73	= 0.40
	AS-AGS3 vs. SC-AGS3	(1, 20) = 0.74	= 0.40
Sucrose	AS-AGS3 vs. SC-AGS3	(1, 8) = 2.00	= 0.20
	AS-AGS3 24 h vs. AS-AGS3 3 wk sucrose	(1, 40) = 1.85	= 0.18
	Deprivation	(1, 40) = 1.39	= 0.25
	Deprivation × knockdown	(1, 40) = 0.10	= 0.76
β ARKct	β ARKct vs. GFP	(1, 12) = 4.98	< 0.05
	β ARKct vs. 24 h depr	(2, 36) = 0.88	= 0.42
	β ARKct vs. 3 wk depr	(1, 24) = 4.23	< 0.05
Giα	AS-Giα_1 vs. SC-pan Giα	(1, 17) = 0.32	= 0.58
	AS-Giα_2 vs. SC-pan Giα	(1, 14) = 0.22	= 0.64
6 wk deprivation			
Multiple testing	Mult. test vs. 24 h depr	(1, 32) = 0.47	= 0.50
	Mult. test vs. 3 wk depr	(1, 32) = 8.98	< 0.01
Single test	6 wk depr vs. 24 h	(1, 34) = 6.72	< 0.05
	6 wk depr vs. 3 wk	(1, 34) = 0.24	= 0.63
3 wk older	Older 24 h depr vs. 24 h	(1, 31) = 0.64	= 0.43
	Cumulative responding		
No virus	3 wk to 24 h depr	(1, 34) = 7.98	< 0.01
	Depr × time	(59, 2,006) = 6.50	< 0.01
Barrier	3 wk barrier vs. 24 h depr	(1, 25) = 3.39	= 0.78
	Depr × time	(59, 1,475) = 7.33	< 0.01
	3 wk barrier vs. 3 wk depr	(1, 25) = 0.43	= 0.52
	Depr × time	(59, 1,475) = 0.17	= 1.0
No-cue	3 wk depr no-cue vs. 24 h	(1, 26) = 0.01	= 0.92
	Depr × time	(59, 1,534) = 0.53	= 1.0
	3 wk depr no-cue vs. 3 wk barrier	(1, 17) = 3.91	= 0.06
	Depr × time	(59, 1,003) = 8.69	< 0.01
AGS3 knockdown			
EtOH	AS-AGS3 vs. SC-AGS3	(1, 26) = 6.84	< 0.01
	Transgene × time	(59, 1,534) = 7.36	< 0.01
	AS-AGS3 vs. 24 h depr	(1, 29) = 0.05	= 0.83
	Transgene × time	(59, 1,711) = 0.83	= 0.81
	SC-AGS3 vs. 3 wk depr	(1, 31) = 0.01	= 0.95
	Transgene × time	(59, 1,829) = 0.35	= 1.0
Sucrose	AS-AGS3 vs. SC-AGS3	(1, 32) = 1.71	= 0.20
	Transgene × time	(59, 1,888) = 2.37	< 0.01
β ARKct	β ARKct vs. GFP	(1, 12) = 3.23	= 0.09
	Transgene × time	(59, 708) = 3.29	< 0.01

	Comparison	*F*	*P*
	Interresponse interval < 5 sec		
No virus	3 wk to 24 h depr	(1, 34) = 7.73	< 0.01
	Depr × time	(3, 102) = 7.17	< 0.01
AGS3 knockdown			
EtOH	AS-AGS3 vs. SC-AGS3	(1, 26) = 6.22	< 0.05
	Transgene × time	(3, 78) = 3.44	< 0.05
β ARKct	β ARKct vs. GFP	(1, 12) = 3.99	= 0.06
	Transgene × time	(3, 36) = 3.18	< 0.05
	Interresponse interval > 10 sec		
No virus	3 wk to 24 h depr	(1, 34) = 6.83	< 0.01
	Depr × time	(89, 3,026) = 2.56	< 0.01
AGS3 knockdown			
EtOH	AS-AGS3 vs. SC-AGS3	(1, 26) = 6.32	< 0.05
	Transgene × time	(89, 2,314) = 6.08	< 0.01
β ARKct	β ARKct vs. GFP	(1, 12) = 2.91	= 0.11
	Transgene × time	(89, 1,068) = 2.32	< 0.05
	Percent interresponse interval		
No virus	3 wk to 24 h depr	(1, 34) = 0.44	= 0.51
	Depr × time	(9, 306) = 0.77	= 0.64
AGS3 knockdown	AS-AGS3 vs. SC-AGS3	(1, 26) = 3.68	= 0.07
	Transgene × time	(9, 234) = 0.71	= 0.70
β ARKct	β ARKct vs. GFP	(1, 12) = 1.19	= 0.30
	Transgene × time	(9, 108) = 0.78	= 0.63