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Antisense-induced knockdown of CREB-binding protein (CBP) downregulates Per1 gene expression in the shell region of nucleus accumbens resulting in reduced alcohol consumption in mice

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Abstract

INTRODUCTION: We have recently shown that circadian genes in the shell region of nucleus accumbens (NAcSh) play a key role in alcohol consumption, however, the exact molecular mechanism is unclear. Since CREB-binding protein (CBP) promotes Per1 gene expression, we hypothesize that alcohol consumption will increase CBP expression in the NAcSh and antisense-induced knockdown of CBP will reduce Per1 expression resulting in a reduction in alcohol consumption.

METHODS: To test our hypothesis, we performed two experiments. Drinking-in-the-dark (DID) paradigm, was used to evaluate alcohol consumption in male C57BL/6J mice. Experiment 1 determined the effects of alcohol consumption on CBP gene expression in the NAcSh. Control experiments were performed by exposing the animals to sucrose [10% (w/v taste and calorie] and water (consummatory behavior). Experiment 2 determined the effects of CBP gene silencing on the expression of the Per1 gene in the NAcSh and alcohol consumption in mice exposed to alcohol using the DID paradigm. CBP gene silencing was achieved by local infusion of two doses of either, CBP antisense oligodeoxynucleotides (AS-ODNs; Antisense group) or nonsense ODNs (NS-ODNs; Nonsense group), bilaterally microinjected in the NAcSh within 24 h before alcohol consumption on Day 4 of the DID paradigm. On completion, histological verification of microinfusion sites was performed by cresyl violet staining.

RESULTS: Compared to sucrose, alcohol consumption, under the DID paradigm, significantly increased the gene expression of CBP in the NAcSh. Compared to the Controls, bilateral infusion of CBP AS-ODNs significantly reduced the gene expression of Per1 in the NAcSh as well as alcohol consumption without affecting the amount of sucrose consumed.

CONCLUSIONS: Our results suggest that CBP is an upstream regulator of Per1 in the NAcSh and may act via Per1 to modulate alcohol consumption.

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CONFLICT OF INTERESTS: None

Keywords

CREB-binding protein; Per1; Nucleus accumbens; Binge drinking; Alcohol

INTRODUCTION

Alcohol use disorders (AUD), as described in the DSM-5, is a chronic relapsing brain disorder responsible for an average of 88,000 deaths each year [reviewed in (Kranzler and Soyka, 2018)]. A person's risk for developing AUD depends, in part, on how much, how often, and how quickly they consume alcohol (NIAAA, 2020). Drinking excessively, which includes frequent binge drinking episodes, over time increases the risk of AUD (Kuntsche et al., 2017). In 2010, the estimated alcohol-related costs in the U.S. were $249 billion, 77% of which was attributable to heavy alcohol consumption (Kranzler and Soyka, 2018). Thus, there is an imperative need to understand the mechanism responsible for alcohol consumption.

We have recently shown that circadian genes present in the shell region of (NAcSh) play a key role in alcohol consumption. While alcohol consumption increases circadian genes expression in the NAcSh, antisense-induced downregulation of these circadian genes reduced alcohol consumption (Sharma et al., 2021). However, the exact molecular mechanism by which alcohol induces an increase in circadian genes expression is unknown.

The CREB-binding protein (CBP) is recruited by cAMP response element binding protein (CREB) and functions as a transcriptional co-activator with histone acetyltransferase (HAT) activity. It has been suggested that CBP is involved in Per1 transcription by promoting transactivation of CLOCK–BMAL1 into the nucleus (Curtis et al., 2004, Lee et al., 2015, Yujnovsky et al., 2006). In addition, overexpression of CBP greatly potentiated the CLOCK-BMAL1-mediated Per1 transcription suggesting a causal relationship between CBP and Per1 gene expression (Lee et al., 2010).

CBP is also implicated to play a role in alcohol-associated behaviors. For example, acute alcohol increases the expression of CBP in the brain and mediates alcohol-induced behaviors such as anxiety [reviewed in (Krishnan et al., 2014)]. Hence, we asked two questions. 1) Does alcohol consumption increase CBP expression? 2) Does CBP modulate the expression of Per1 in the NAcSh to promote alcohol consumption? Thus, we hypothesized that a) alcohol consumption will increase the expression of CBP in the NAcSh and b) Antisense-induced knockdown of the CBP gene in the NAcSh will downregulate Per1 gene expression and will reduce alcohol consumption.

MATERIALS AND METHODS

Animals:

Male C57BL/6J mice (RRID: IMSR_JAX:000664), aged 7 to 8 weeks, were housed in a group of four/cage in the vivarium, located at Harry S. Truman Memorial Veterans (HSTMV) Hospital, with room temperature maintained at 25 ± 2°C and a light-dark cycle (12 h – 12 h; light onset at 7 AM). The animals had ad libitum access to food and water.

Prior to any experimental procedure, animals were moved to the experiment room which had a reverse light-dark cycle (lights on at 2 AM) and was allowed to acclimate to the new environment for at least 14 days. All experiments performed in this study comply with the AAALAC and Institute for Laboratory Animal Research. The protocols used in this study were approved by the local committee at HSTMV Hospital (SAS#245).

Chemicals:

Ethanol (200 proof; Fisher Scientific, Pittsburgh, PA) was used to prepare alcohol solution (20% v/v in tap water), D-Sucrose (Sucrose; Fisher Scientific) was used to prepare sucrose solution (10% w/v in tap water). For gene silencing, the phosphorothioate antisense oligonucleotide (AS-ODN) and nonsense oligonucleotides (NS-ODN) were procured from Integrated DNA Technologies (IDT; Coralville, IA, USA). The description of AS-ODN and NS-ODN sequences is provided in Table 1. Once received, the AS-ODNs or NS-ODNs were reconstituted to prepare a stock solution (10X). From these stock solutions, working solutions were prepared fresh before administration. For RT-PCR, the primers for CBP [designed using online software (Untergasser et al., 2012); see Table 1] and Per1 [described earlier (Sharma et al., 2021)] were obtained from IDT.

Alcohol consumption:

Animals were exposed to four-day drinking-in-the-dark (DID) procedure, a widely accepted model to facilitate excessive alcohol consumption in non-stressful (home cage) environment, as described earlier (Sharma et al., 2014a, Rhodes et al., 2005, Thiele and Navarro, 2014, Sharma et al., 2021). In brief, on Day 1, after 2.5 h of dark onset, the water bottle was removed from the mouse cage. After 30 min, a pre-weighed bottle (identical to water bottle) filled with alcohol [20% (v/v)] was presented to the mouse in place of a water bottle. The mouse was allowed to self-administer alcohol for 2 h. After alcohol consumption, alcohol bottle was removed and weighed, followed by weighing the animals and replacing the original water bottle. The amount of alcohol consumed (g/kg of the body weight) was calculated and the mouse was left undisturbed. This procedure was repeated for the next 2 days (Day 2 and 3). On day 4, the animal was again exposed to alcohol as described above except this time, the alcohol consumption was continued for 4 h. In control experiments, animals were exposed to sucrose [10% (w/v); control for taste and calorie] or water (control for consummatory behavior) using the DID procedure as described above.

Experimental paradigm:

The animals were randomly assigned as previously described (Sharma et al., 2021). However, the experimenters were blinded to the treatment conditions. The control and experimental groups were run in parallel and repeated for at least two times in order to achieve rigor and reproducibility. We performed a *priori* analysis [$\alpha = 0.05$; power ≥ 0.9] after preliminary experiments (N=3/group) to calculate the effect and sample size using G*Power (Faul et al., 2007).

Experiment 1: Effect of alcohol consumption on CBP gene expression in the accumbal shell region (NAcSh).—Our preliminary experiments followed by G*Power analysis (F test) suggested an effect size of 0.99 and a total of 21 mice (7/Group). A total of

21 mice were used in this experiment. They were randomly assigned to the following three groups: Alcohol (N = 7), Sucrose (N = 7), and Water (N = 7).

Alcohol consumption: As described above, on Days 1 to 3, animals were allowed to self-administer either alcohol (Alcohol group), sucrose (Sucrose group) or water (Water group) for 2 h. On Day 4, animals were exposed to alcohol for 4 h and subsequently euthanized by decapitation within an hour.

RT-PCR: After euthanization, the brains were rapidly removed. The accumbal shell region [Anterior (from +1.18 to +1.70 mm), lateral (±0.5 mm), and ventral (from −4.5 to −5.0 mm); (Franklin and Paxinos, 2008)] was punched out and the tissue was processed for RNA isolation and RT-PCR to determine the gene expression of CBP as described earlier (Sharma et al., 2010, Sharma et al., 2018, Sharma et al., 2021).

Experiment 2: Effect of CBP gene knockdown in the NAcSh on alcohol consumption—Our preliminary experiments followed by G*Power analysis (F test) suggested an effect size of 0.99 and a total of 21 mice (7/Group). A total of 28 mice were used in this experiment to determine the effect of CBP antisense infusion in the NAcSh on alcohol (N =14; N = 7/group) and sucrose (Control for taste and calorie; N = 14; N = 7/group) consumption.

Surgery: The cannulas for bilateral infusion of CBP antisense were implanted in the NAcSh as described earlier (Thakkar et al., 2010, Sharma et al., 2014a, Sharma et al., 2015, Sharma et al., 2021). Briefly, using isoflurane (2%) anesthesia and under aseptic conditions, stereotaxic surgeries were performed to implant guide cannulas (25 G) targeted towards the NAcSh using the following coordinates: Anterior (+1.4 mm), lateral (±0.5 mm), and ventral (−4.8 mm); relative to bregma and the skull surface (Franklin and Paxinos, 2008). Once the guides were implanted (2.0 mm above the NAcSh), they were fixed to the skull by using metal screws and dental cement. Flunixin, an analgesic, was administered twice in 24 h (2.5 mg/kg) after the surgery. Thereafter, the animals, once ambulatory, were housed singly in the experimental cage [home cage with one grommeted hole on the front (shorter) side] with a water bottle presented from the sides. The animals were left undisturbed to recover from surgical stress for 5 to 7 days.

Habituation: To reduce the handling stress, sham microinjections were performed on Days 1, 2, and 3 in mice exposed to the DID paradigm (described above). During sham microinjection, the sham-injector, 1.5 mm shorter than the actual microinjector cannula, was inserted into the guide cannula without injecting any fluid. On completion, the animals were returned to their cages.

a) Alcohol consumption: After habituation, the animals (N = 14) were divided into two [AS-ODN (Antisense; N = 7) and NS-ODN (Control; N = 7)] groups. Animals were bilaterally microinjected with CBP antisense [AS-ODN group; 0.5 μg AS-ODNs dissolved in 500 nl 0.9% saline] or NS-ODNs (Control group; 500 nl) into the NAcSh (described above). Two doses of CBP antisense were administered bilaterally in the NAcSh; One at 21 h and the second at 9 h prior to alcohol consumption on Day 4. The microinjection

procedure used in this study was described earlier (Thakkar et al., 2010, Sharma et al., 2014a, Sharma et al., 2015).

Blood Alcohol Concentration (BAC): On Day 4, after measuring the amount of alcohol consumption (described above), the tail vein blood was withdrawn to examine the BAC (Sharma et al., 2014b).

b) Sucrose consumption: Similar to alcohol consumption, the effect of CBP gene knockdown in the NAcSh on sucrose consumption was determined in a separate group of animals, using the DID paradigm.

Histological verification of microinjection sites: After alcohol/sucrose consumption on Day 4, animals were deeply anesthetized using CO_2 anesthesia followed by transcardial perfusion with ice-cold normal saline and subsequently with a fixative (10% buffered formalin; Fisher Scientific). The brains were isolated, cut into thin sections (30 μm) and stained with cresyl violet to verify the bilateral microinjection site in the accumbal shell region of the brain (Sharma et al., 2010, Sharma et al., 2014c).

c) Per1 gene expression following CBP antisense infusion in the NAcSh: Next, we verified the downregulation of CBP gene and examined the expression of Per1 gene following microinjection of CBP antisense in the NAcSh using RT-PCR. The animals were exposed to alcohol using the DID paradigm along with habituation with sham microinjections on Days 1 to 3 as described above. On Day 4, mice (N = 14) were assigned to two [Control (N = 7) and Antisense (N = 7)] groups. The Controls were administered with NS-ODNs and animals in the Antisense group were microinfused with CBP AS-ODNs into the accumbal shell region of the brain as described above. Next, the animals were exposed to alcohol for 4 h. After alcohol consumption, animals were euthanized, brains removed and sectioned into thin slices using brain slicer. After visual verification of microinjection sites in the NAcSh, the accumbal shell region was punched out as described above. The tissue was processed for RT-PCR to examine the gene expression of CBP and Per1 (Sharma et al., 2021).

Statistics: In all the experiments, the statistical analysis was performed by using “R” statistics (version 3.6.3) (R-CoreTeam, 2013). The online Graphpad’s Outlier calculator (Grubb’s) was used to determine the possible outlier. The Kolmogorov-Smirnov test was used to assess the normality of data.

One-Way ANOVA was performed to determine a) the changes in the CBP gene expression in the NAcSh following alcohol, sucrose, and water consumption on Day 4 of the DID paradigm. Unpaired t-test was used to analyze a) the changes in the alcohol and sucrose consumption following antisense-induced knockdown of CBP gene expression in the NAcSh and b) the changes in local gene expression of CBP and Per1 following CBP antisense in the NAcSh. Two-way repeated measure (RM) ANOVA was used to analyze the changes in alcohol/sucrose consumption with treatment as a between-group variable (Two levels: Control and Antisense) and time as a within-group variable (Three levels: Day 1 to Day 3). The level of significance (α) was kept at 0.05.

RESULTS

Experiment 1: CBP gene expression was increased in the NAcSh after alcohol consumption on Day 4 of the DID paradigm.

A. Drinking: On Days 1 to 3, no significant change was observed in water ($F_{2,18} = 0.63$, $p > 0.05$; N = 7), sucrose ($F_{2,18} = 1.31$, $p > 0.05$; N = 7) and alcohol ($F_{2,20} = 1.89$, $p > 0.05$; N = 7) consumption across days. The data is described in Table 2. On Day 4, the mean ± SEM amount of water, sucrose, alcohol consumption and BAC was 35.42 ± 1.49, 147.56 ± 8.03, 5.51 ± 0.14 and 110.65 ± 6.74 mg/dl (N = 7/group) in mice. The BAC was significantly ($R^2 = 0.88$; $p < 0.01$) correlated with the amount of alcohol consumed.

B. Gene expression: One-Way ANOVA suggested a significant ($F_{2,20} = 13.85$; $p < 0.001$) effect of treatment on the gene expression of CBP in the NAcSh of mice. Newman-keuls post hoc test suggested that as compared to mice exposed to sucrose (N = 7), mice exposed to alcohol displayed a significant ($p < 0.001$; N = 7) increase in the CBP gene expression in the NAcSh. No significant difference was observed between water (N = 7) and sucrose (N = 7) groups (Figure 1D).

Experiment 2: The CBP gene Knockdown in the NAcSh reduces alcohol consumption in mice.

Our preliminary experiments followed by G*Power analysis (unpaired t-test) suggested an effect size of 2.7 and a total of 14 mice (7/Group).

A. Effect of CBP knockdown in the NAcSh on Alcohol consumption.

Microinjection sites: A total of 14 animals were used in this experiment. Histological assessment of brain sections suggested that all microinjection sites [N = 14 (7/group); indicated as black (NS-ODN group) and red (AS-ODN group) circles] were localized in the NAcSh region (between AP = +1.5 mm and +1.1 mm; relative to bregma). All bilateral microinjection sites (N = 14) were mapped on one single coronal schematic [(AP = 1.2; adapted from Figure 21; see Franklin and Paxinos (2008)] and described in Figure 2A. Additionally, a representative photomicrograph with bilateral injection sites in the NAcSh is described in Figure 2B.

Alcohol consumption: On Days 1 to 3, alcohol consumption was comparable between Control and Antisense groups as we did not find any significant main effect of treatment ($F_{1, 24} = 0.13$; $p > 0.05$; N = 7), time ($F_{2, 24} = 2.74$; $p > 0.05$; N = 7) and interaction ($F_{2, 24} = 1.35$; $p > 0.05$; N = 7) on alcohol intake. The mean ± SEM alcohol consumption was 2.56 ± 0.16 (Day 1), 2.70 ± 0.11 (Day 2) and 2.95 ± 0.12 (Day 3) g/Kg in the Antisense group whereas 2.70 ± 0.12 (Day 1), 2.63 ± 0.08 (Day 2) and 2.76 ± 0.06 (Day 3) g/Kg in the Control group.

On Day 4, CBP antisense microinfusion in the NAcSh significantly ($t = 3.75$; $df = 12$, $p < 0.01$; unpaired t-test) reduced alcohol consumption (3.54 ± 0.16 g/Kg; N = 7) in comparison to the control treatment (4.28 ± 0.11 g/Kg; N = 7) (Figure 2C).

BAC: As compared to the mice administered with NS-ODN (104.03 ± 4.64 mg/dl; N = 7), mice administered with AS-ODN displayed a significant ($t = 3.99$, $df = 12$, $p < 0.01$; unpaired t-test) reduction in the BAC (74.58 ± 5.72 mg/dl; N = 7) values (Figure 2D).

B. Knockdown of CBP gene in the NAcSh did not affect sucrose consumption

Microinjection sites: In this experiment, 14 animals were used. Histological mapping of all microinjection sites from CV-stained brain sections of animals in NS-ODN group (N = 7; indicated as black circles) and AS-ODN group (N = 7; indicated as red circles) suggested that all sites were found in the NAcSh region (between AP = +1.5 mm and +1.1 mm; relative to the bregma).

All bilateral microinjection sites (N = 14) were mapped on one single coronal schematic [(AP = 1.2; adapted from Figure 21; see Franklin and Paxinos (2008)] and described in Figure 3A. Additionally, a representative photomicrograph with bilateral injection sites in the NAcSh is described in Figure 3B.

Sucrose consumption: Two-Way RM ANOVA did not show any significant main effect of treatment ($F_{1, 24} = 0.44$; $p > 0.05$), time ($F_{2, 24} = 1.10$; $p > 0.05$) and interaction ($F_{2, 24} = 0.25$; $p > 0.05$) on sucrose consumption across Days 1 to 3. The mean ± SEM amount of sucrose consumed on was 9.28 ± 0.36 (Day 1), 9.30 ± 0.20 (Day 2) and 9.52 ± 0.23 (Day 3) g/Kg in mice in the Antisense group whereas 9.21 ± 0.33 (Day 1), 9.27 ± 0.33 (Day 2) and 9.89 ± 0.28 (Day 3) g/Kg in the Control group.

On Day 4, mice treated with AS-ODN did not display any significant ($t = 0.05$; $df = 12$, $p > 0.05$; unpaired t-test) change in sucrose consumption as compared to the Controls. The mean ± SEM consumption of sucrose was (12.22 ± 0.15 g/kg; N = 7) in AS-ODN treated mice and 12.22 ± 0.15 g/kg (N = 7) in NS-ODN treated mice (Figure 3C).

C. Infusion of CBP antisense downregulates CBP and Per1 genes in the NAcSh.

Alcohol consumption: On Days 1 to 3, alcohol consumption was comparable between Control and Antisense groups (Data not shown). On day 4, CBP antisense microinfusion in the NAcSh significantly ($t = 3.24$; $df = 12$, $p < 0.01$; unpaired t-test) reduced alcohol consumption (3.76 ± 0.08 g/Kg; N = 7) as compared to the control treatment (4.18 ± 0.10 g/Kg; N = 7).

BAC: As compared to the mice administered with NS-ODN (97.49 ± 3.05 mg/dl; N = 7), mice administered with AS-ODN displayed a significant ($t = 2.59$, $df = 12$, $p < 0.01$; unpaired t-test) reduction in the BAC (86.10 ± 6.33 mg/dl; N = 7).

CBP: Mice in the Antisense group (N = 7) showed a significant ($p < 0.05$) downregulation of CBP ($t = 3.75$; $df = 12$; unpaired t-test; Figure 4A) gene as compared to mice in the Control group (N = 7).

Per1: Mice in the Antisense group (N = 7) showed a significant ($p < 0.05$) downregulation of Per1 ($t = 3.60$; $df = 12$; unpaired t-test; Figure 4B) gene as compared to mice in the Control group (N = 7).

DISCUSSION

Recently, we have shown that circadian genes in the NAcSh are responsible for alcohol consumption (Sharma et al., 2021). In this study, we performed two experiments in C57BL/6J mice to investigate the role of CBP, a transcriptional co-activator, in the NAcSh, in alcohol consumption. The major findings of the study are that a) gene expression of CBP was significantly increased in the NAcSh after four hours of alcohol consumption on Day 4 in mice exposed to DID paradigm, b) knockdown of CBP genes in the NAcSh using antisense technique significantly reduced alcohol consumption without affecting sucrose consumption and c) infusion of CBP antisense in the NAcSh caused a significant reduction in the expression of Per1. These results suggest that CBP is an upstream regulator of Per1 expression and promotes alcohol consumption in the NAcSh region.

We used the “DID paradigm”, a widely used method to understand the neurobiology of alcohol consumption [reviewed in (Thiele and Navarro, 2014)]. In this method, while sucrose and other fluid consumption increases similar to the alcohol on Day 4, relative to Days 1–3, C57BL/6J mice, exposed to 20% alcohol, voluntarily administer significant amounts of alcohol on Day 4 resulting in high BAC which corresponds to BAC observed after binge drinking in humans (Gowin et al., 2017).

The accumbal shell region of nucleus accumbens has been implicated in motivation for reward and its reinforcement (McBride et al., 1999, Clarke and Adermark, 2015, Di Chiara, 2002). Within the NAcSh, the medial NAcSh plays a crucial role in alcohol consumption (Lei et al., 2019, Kasten and Boehm, 2014, Cozzoli et al., 2012, Balla et al., 2018). Indeed, our previous findings suggested that while downregulation of circadian genes in the NAcSh reduced alcohol consumption, no such effect was observed by knockdown of circadian genes in the core region of the nucleus accumbens (Sharma et al., 2021). Hence, in the present study, we targeted the NAcSh region for investigating the molecular mechanism implicating Per1 in the regulation of alcohol consumption.

Our first experiment was focused on CBP expression in the NAcSh after alcohol consumption because clinical and preclinical studies suggest that CBP is involved in alcohol addiction (Sterner and Berger, 2000, Levine et al., 2005, Ghezzi et al., 2017, Malvaez et al., 2011, Noda and Nabeshima, 2000). For example, in animals, increased neuronal activity results in CREB phosphorylation and nuclear translocation where it recruits CBP which may be associated with behavioral effects of alcohol exposure such as anxiety, motor incoordination, associated with acute and chronic alcohol consumption (Teppen et al., 2016, Zhang et al., 2018, Guo et al., 2011). Clinical studies also suggest that single nucleotide polymorphism in the CBP gene is associated with alcohol addiction (Kumar et al., 2011). In addition, CBP is also linked to the development of addiction towards other substance use. For example, mice that are haploinsufficient for CBP show decreased sensitivity to cocaine

(Levine et al., 2005). Furthermore, focal knock-out of CBP in the nucleus accumbens reduces cocaine sensitivity and reward (Malvaez et al., 2011).

We found that alcohol consumption on Day 4 of the DID paradigm leads to an increased expression of CBP in the NAcSh. The results are congruent to previous *in vitro* and *in vivo* studies suggesting increased CBP expression after alcohol exposure (Constantinescu et al., 2004, Dulman et al., 2019). In our second experiment, we chose to examine the expression of Per1 in the NAcSh after CBP knockdown because overexpression of CBP greatly potentiates CLOCK-BMAL1-mediated Per1 transcription (Lee et al., 2010, Curtis et al., 2004, Lee et al., 2015, Yujnovsky et al., 2006).

We used the “antisense technique”, which has been extensively used to study the functional role of genes in animals and humans [reviewed in (Schoch and Miller, 2017)]. This technique is highly specific for inhibiting genes in targeted brain regions. The AS-ODN sequence used in this study has 100% homology with mouse CBP and its selectivity in the knockdown of CBP has been validated by several studies (Saha et al., 2009, Auger et al., 2002, Molenda et al., 2002). The NS-ODNs sequence had no sequence homology to any known genes in mice as verified by BLAST tool (Altschul et al., 1990). We administered two doses of CBP antisense ODNs, within 24 h before alcohol consumption on Day 4, to achieve maximal knockdown of CBP expression (Saha et al., 2009, Auger et al., 2002, Molenda et al., 2002).

Our results suggest that antisense-induced knockdown of CBP in the NAcSh reduced binge alcohol consumption as revealed by a significant ($p < 0.003$) reduction (>18%) in the alcohol consumption and in the BAC values (>29%). In addition, we also found that antisense-induced downregulation of CBP caused a significant reduction in the Per1 gene expression in the NAcSh suggesting that CBP may promote alcohol consumption via Per1 (Sharma et al., 2021). While this seems to be a mechanism for binge drinking, further studies are required to investigate the role of CBP in NAcSh in other methods of alcohol consumption (intermittent alcohol 2-bottle choice) in mice.

In summary, our results suggest that alcohol consumption increases CBP gene expression in the NAcSh. Local antisense-induced knockdown of CBP significantly attenuated the Per1 gene expression in the NAcSh resulting in a significant reduction in alcohol consumption. This suggests that CBP may act as an upstream regulator of Per1 gene expression to promote alcohol consumption.

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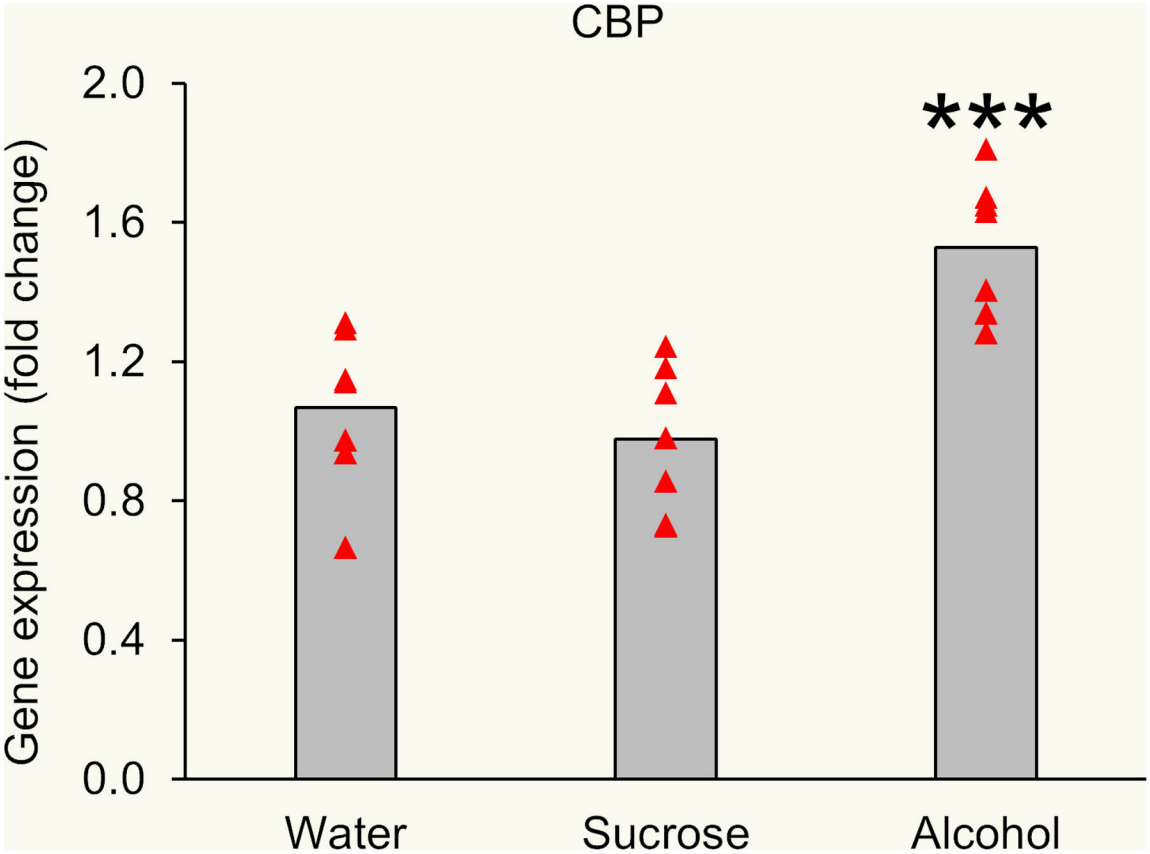

Figure 1: Effect of alcohol consumption on CBP gene expression.

On Day 4, alcohol consumption for 4 h caused a significant increase in CBP gene expression in the accumbal shell region (NAcSh) in mice (N = 7) as compared to the sucrose consumption (Control group; N = 7). No significant difference was observed in CBP gene expression in mice exposed to sucrose and water. ***p < 0.001 vs Sucrose.

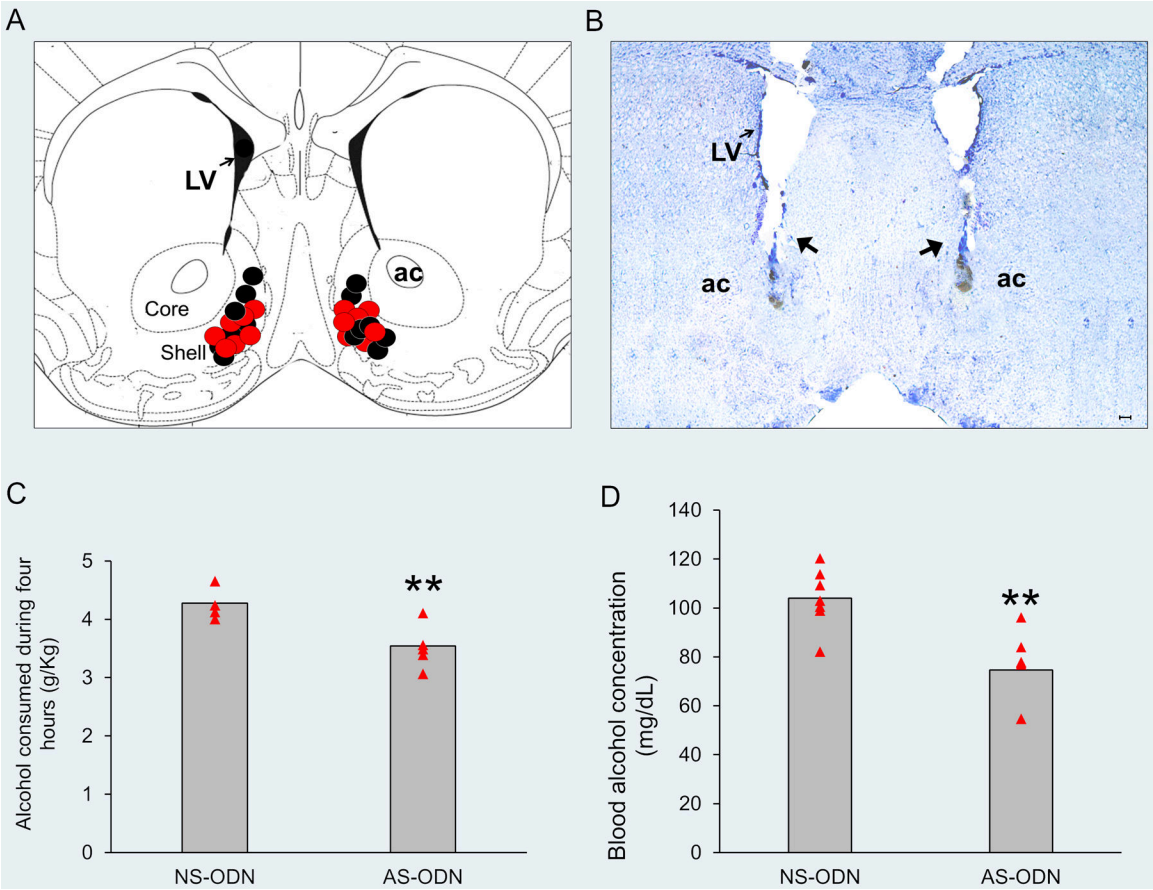

Figure 2: Effect of knockdown of CBP in the accumbal shell region on alcohol consumption in mice.

Panel A: The lesions caused due to bilateral microinjections of CBP antisense oligodeoxynucleotides (AS-ODN; Antisense Group; N = 7; Red circles) or nonsense oligodeoxynucleotides (NS-ODN; Control Group; N = 7; black circles) in the accumbal shell region (NAcSh) of mice, localized between 1.5 and 1.1 mm anteroposterior, is depicted in a schematic coronal brain section (Plate 21, AP = 1.2 mm, (Franklin and Paxinos, 2008)).

Panel B: Lesions (black arrows) caused due to insertion of microinjector cannula in the accumbal shell region is described in a representative photomicrograph. Scale bar = 100 μm.

Panel C: Compared to nonsense oligodeoxynucleotide (NS-ODNs; N = 7), bilateral infusion of CBP antisense oligodeoxynucleotide (AS-ODNs; N = 7) in the accumbal shell region caused a significant reduction in alcohol consumption during 4 h of alcohol access on day 4 of the DID paradigm. **$p < 0.01$.

Panel D: As compared to the mice treated with NS-ODN (Controls; N = 7), mice bilaterally infused with CBP AS-ODN (Antisense; N = 7) in the accumbal shell region showed a significant reduction in blood alcohol concentration after 4 h of alcohol access on Day 4. **$p < 0.01$ vs NS-ODN. Abbreviations: LV, lateral ventricles and ac, anterior commissure.

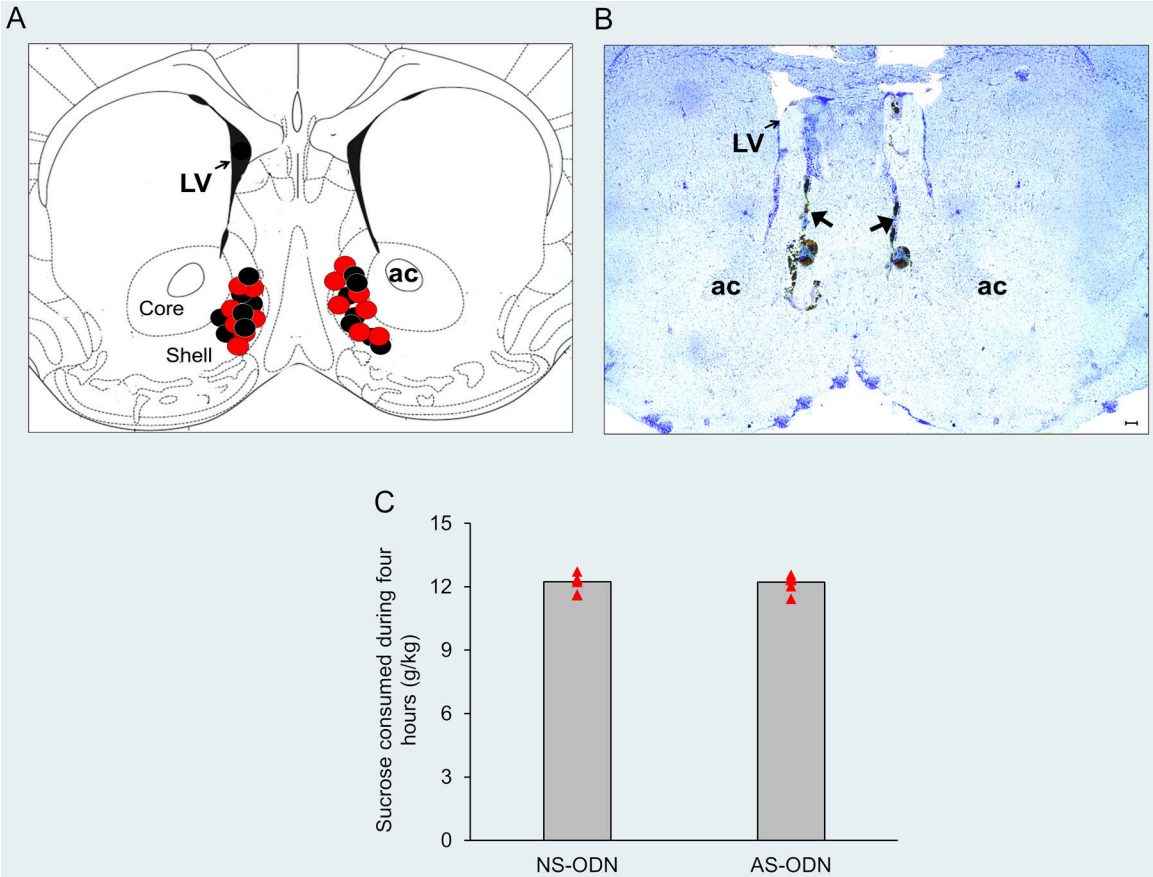

Figure 3: Effect of CBP gene knockdown in the accumbal shell region (NAcSh) on sucrose consumption.

Panel A: A coronal schematic showing the lesions (localized between 1.5 and 1.1 mm anteroposterior) caused due to bilateral microinjections of CBP antisense oligodeoxynucleotides (AS-ODN; Antisense Group; N = 7; Red circles) or nonsense oligodeoxynucleotides (NS-ODN; Control Group; N = 7; black circles) in the accumbal shell region (NAcSh) of mice [Figure 21, 1.2 mm anteroposterior; (Franklin and Paxinos, 2008)].

Panel B: A representative photomicrograph depicting the bilateral microinjector cannula insertion sites in the accumbal shell region (denoted by black arrows). Scale bar = 100 μm.

Panel C: No significant change was observed in sucrose consumption during 4 h access to sucrose (10%) solution using the DID paradigm in mice infused with CBP AS-ODN in accumbal shell region (Antisense group; N = 7) compared to the mice treated with NS-ODN (Controls; N = 7) on Day 4. Abbreviations: LV, lateral ventricles and ac, anterior commissure.

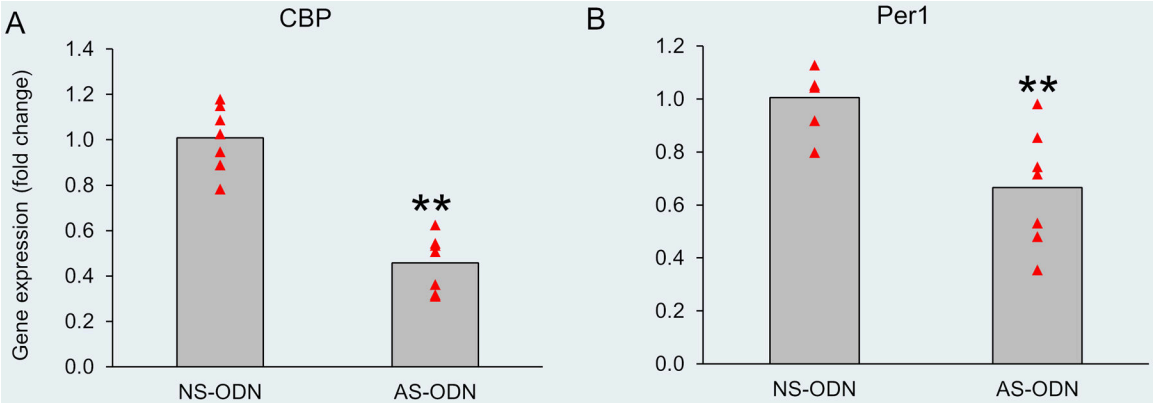

Figure 4: Effect of bilateral administration of a CBP antisense oligodeoxynucleotide on CBP and Per1 gene expression.

Microinfusion of CBP antisense oligodeoxynucleotide (AS-ODN; Antisense group; N = 7) caused a significant downregulation in the expression of CBP (**Panel A**) and Per1 (**Panel B**) genes into the accumbal shell region as compared to the nonsense oligodeoxynucleotide (NS-ODN; Control group; N = 7). **p < 0.01 vs NS-ODN.

Table 1:

List of CBP primers and oligodeoxynucleotide

		Gene Accession #
Forward Primer	TGT GCC CTT CTG CCT CAA CA	NM_001025432.1
Reverse Primer	GTG GTG TTT GGG GTG TGC TG	
AS-ODN	CAG*CAA GTT CTC GGC CAT*CTT	
NS-ODN	CGA*TGC GTC ATC GAC*CAT	

*Indicates phosphorothioate linkage within the oligonucleotide (ODN) sequence. AS-ODN = Antisense oligodeoxynucleotide; NS-ODN = Nonsense oligodeoxynucleotide

Table 2:

Amount of fluid consumption (g/kg) during 2 h of access to water, sucrose and alcohol on days 1–3 of the DID paradigm

Days	Water	Sucrose	Alcohol
1	24.40 ± 0.80	94.54 ± 2.81	3.63 ± 0.09
2	22.97 ± 0.91	98.64 ± 2.39	3.82 ± 0.12
3	24.26 ± 1.11	101.47 ± 2.43	3.90 ± 0.14