HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2023 July 13.

Published in final edited form as:

Neuropharmacology. 2022 July 01; 212: 109063. doi:10.1016/j.neuropharm.2022.109063.

Pituitary adenylate cyclase-activating polypeptide type 1 receptor within the nucleus accumbens core mediates excessive alcohol drinking in alcohol-preferring rats

Margaret A. Minnig[a,1], **Angelo Blasio**[a,1], **Antonio Ferragud**[a], **Yasmine N. Sami**[a], **Emily E. Erhard**[a], **Rose H. Clark**[a], **Alyssa DiLeo**[a], **Chiara Giuliano**[b], **Barry J. Everitt**[b], **Pietro Cottone**[a], **Valentina Sabino**[a,*]

[a]Laboratory of Addictive Disorders, Departments of Pharmacology and Experimental Therapeutics and Psychiatry, Boston University School of Medicine, Boston, MA, USA

[b]Behavioral and Clinical Neuroscience Institute and Department of Psychology, University of Cambridge, Cambridge, UK

Abstract

Alcohol use disorders (AUD) have a strong component of heritability; however, the neurobiological mechanisms mediating the propensity to consume excessive amounts of alcohol are still not well understood. Pituitary adenylate cyclase-activating polypeptide (PACAP), a highly conserved neuropeptide which exerts its effects mainly through the PAC1 receptor (PAC1R), has been suggested to be one of the mediators of the effects of drugs of abuse and alcohol. Here, we investigated the role of the PACAP/PAC1R system in excessive alcohol drinking in alcohol-preferring rats, an established animal model of AUD. Intracerebroventricular (i.c.v.) administration of the PAC1R antagonist PACAP(6–38) blocked excessive alcohol drinking and motivation to drink in Sardinian alcohol-preferring (Scr:sP) rats, without affecting water, saccharin, or sucrose intake. Notably, PACAP(6–38) did not affect ethanol responding in outbred Wistar rats. PACAP(6–38) also significantly reduced alcohol-seeking behavior under a second-order schedule of reinforcement. Using immunohistochemistry, a significant increase in the number of PAC1R positive cells was observed selectively in the nucleus accumbens (NAcc) Core of Scr:sP rats, compared to Wistar rats, following alcohol drinking. Finally, excessive drinking in Scr: sP rats was suppressed by intra-NAcc Core, but not intra-NAcc Shell, PACAP(6–38), as well as by virally-mediated PAC1R knockdown in the NAcc Core. The present study shows that hyperactivity of the PACAP/PAC1R system specifically in the NAcc Core mediates excessive drinking of alcohol-preferring rats, and indicates that this system may represent a novel target for the treatment of AUD.

*Corresponding author. Laboratory of Addictive Disorders, Departments of Pharmacology and Experimental Therapeutics and Psychiatry, Boston University School of Medicine, 72 E. Concord St., R-612, Boston, MA, 02118, USA. vsabino@bu.edu (V. Sabino).

[1]These two authors equally contributed to the work.

Author contributions

MAM, AB, AF, CG, BJE, PC and VS designed the experiments; MAM, AB, AF, YNS, EEE, RHC and AD performed the experiments; MAM, AB, AF, PC and VS analyzed the data; MAM and AB wrote a first draft of the manuscript; all authors edited the manuscript and approved the final version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2022.109063.

1. Introduction

Alcohol use disorder (AUD) is a severe and debilitating disorder which results in 3.3 million deaths worldwide each year (Jones et al., 2015; Kranzler and Soyka, 2018). AUD is characterized by uncontrolled heavy drinking as well as craving and seeking (Association, 2013; Domi et al., 2021; Koob and Volkow, 2016a). Its etiology is complex, as heritable susceptibility factors contribute 50–60% of the disease risk and interact with environmental factors that produce and maintain the disorder (Goldman et al., 2005; Jones et al., 2015; Prescott and Kendler, 1999). Furthermore, many of the same molecular correlates of the genetic propensity to drink can also be generated environmentally by ethanol exposure (Bell et al., 2013; Heilig and Koob, 2007; Sabino et al., 2009). In this investigation, a line of alcohol-preferring rats genetically selected for alcohol preference and consumption were used to investigate the neurobiological factors underlying the predisposition to consume excessive quantities of alcohol (Ciccocioppo and Hyytia, 2006; Crabbe, 2014; Gessa, 2016; McBride et al., 2014; Timme et al., 2020).

The focus of the present study was the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptor, PAC1R. Originally isolated from the ovine hypothalamus (Miyata et al., 1989), PACAP is encoded by the *Adcyap1* (PACAP) gene and exists in two different isoforms, PACAP-27 and PACAP-38, the latter representing more than 90% of the PACAP found in the brain (Masuo et al., 1993; Miyata et al., 1989; Piggins et al., 1996). This neuropeptide belongs to a superfamily that includes growth hormone-releasing hormone (GHRH), secretin, and vasoactive intestinal peptide (VIP) (Sherwood et al., 2000). PACAP binds to three different receptors; two of them, VPAC1 and VPAC2, recognize both VIP and PACAP with equally high affinity, while the third, PAC1R, is a PACAP-selective receptor (reviewed in (Harmar et al., 2012)). The PACAP/PAC1R system has been shown to regulate food intake, energy metabolism, body temperature, and neuronal survival (Gray et al., 2002; Hawke et al., 2009; Iemolo et al., 2015; Inglott et al., 2011; Mounien et al., 2009; Resch et al., 2011). Several groups, including ours, have shown that the PACAP system is a strong mediator of the body's multifaceted response to stress (Agarwal et al., 2005; Dore et al., 2013; Hammack et al., 2009, 2010; Iemolo et al., 2016; Jiang and Eiden, 2016; Mustafa et al., 2015; Ressler et al., 2011; Seiglie et al., 2015, 2019; Varodayan et al., 2020).

A growing body of evidence has begun to link the PACAP/PAC1R system with the effects of drugs of abuse and alcohol (Gargiulo et al., 2020a; Miles et al., 2019; Stojakovic et al., 2020). In humans, a single nucleotide polymorphism (SNP) of *Adcyap1*, rs2856966 (Asp54Gly), with potential relevance to alcohol drinking was identified in a Finnish population of social drinkers, such that the recessive model of the GG genotype predisposes individuals to higher levels of intake (Kovanen et al., 2010). Additional evidence from human studies indicates that a PAC1R variant is associated with problematic alcohol use in women (Dragan et al., 2017). Studies on the intoxicating and ataxic effects of ethanol in *Drosophila* led to the identification of a mutant (named "*Cheapdate*") with higher sensitivity to ethanol-induced ataxia, due to an allele of the gene that encodes PACAP (Feany and Quinn, 1995); this suggests that PACAP signaling may mediate the response that produces the acute tolerance to ethanol-induced ataxia, diminishing the intoxicating actions

of ethanol. Since lower impairment during the early stages of drinking is linked to a greater risk of long-term alcohol addiction (Schuckit, 1994, 1998), high amounts of PACAP may lead to a higher preference for self-administering alcohol. Moreover, acute ethanol has been shown to increase PAC1R mRNA levels via Receptor For Activated C Kinase 1 (RACK1) in cell lines (He et al., 2002) and PACAP has also been reported to modulate the hypothermic effects of ethanol (Szabó et al., 1998; Tanaka et al., 2004). A role for the PACAP/PAC1R system of the bed nucleus of the stria terminalis (BNST) in the excessive alcohol intake that results from chronic intermittent alcohol exposure (Ferragud et al., 2021) and an effect of PACAP-27 and PACAP-38 on home cage alcohol drinking (Gargiulo et al., 2020b) have been shown, suggesting a potential key role for this peptide system in the regulation of alcohol drinking.

The nucleus accumbens (NAcc) is a key node in the circuitry mediating reinforcement and some behavioral effects of addictive drugs (Koob, 2014; Koob and Volkow, 2010, 2016b). The loss of PACAP/PAC1R signaling in the NAcc Shell has recently been shown to increase alcohol drinking in the Scr:sP alcohol-preferring rat line used here (Minnig et al., 2021); this suggests that this system in the NAcc Shell provides a brake on alcohol drinking, in line with the notion that corticostriatal projections to this subregion are part of a “stop” circuitry that leads to decreased drug and alcohol seeking (Koob and Volkow, 2016a; LaLumiere et al., 2012). In contrast, the NAcc Core is a mediator of Pavlovian influences on instrumental action, and corticostriatal projections to the NAcc Core pathway are thought to represent a “go” system involved in drug craving (Corbit et al., 2001, 2016; Koob and Volkow, 2016a; LaLumiere et al., 2012). Neuroadaptations in the NAcc Core following alcohol (and drug) consumption over extended periods of time have been shown, suggesting that neuroplasticity in this region may mediate the effects of long-term alcohol (Feduccia et al., 2014; Malinen and Hyytiä, 2008; Willuhn et al., 2014). The NAcc Core expresses high levels of PACAP and PAC1R (Ghatei et al., 1993; Palkovits et al., 1995; Zhang et al., 2021). Whether alterations of the PACAP/PAC1R system contribute to the predisposition to excessively consume alcohol is currently unknown. Here, we hypothesized that PAC1R hyperactivity, specifically in the NAcc Core, is involved in excessive drinking in alcohol-preferring rats.

2. Materials and methods

Please see Supplementary Material for additional details on the procedures used.

2.1. Subjects

Subjects used were male Wistar rats, weighing 225–250 g upon arrival (Charles River, Wilmington, MA, USA), and male rats derived from the TSRI Sardinian alcohol-preferring (sP) rats (Scr:sP, 29–30th generation, http://rgd.mcw.edu/rgdweb/report/strain/main.html?id=2302666) maintained at Boston University without further selective breeding. Rats were housed in an AAALAC-approved humidity- and temperature-controlled vivarium on a 12-h reverse light–dark cycle (lights off at 09:00) with water and regular rodent chow available *ad libitum*. Experiments were conducted during the rats’ dark cycle. Procedures adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and

the Principles of Laboratory Animal Care and were approved by Boston University Medical Campus Institutional Animal Care and Use Committee.

2.2. Drugs and viral constructs

Ethanol solution (10% *w/v*) was prepared using 95% ethyl-alcohol and tap water. Saccharin solution (0.02% *w/v*) was prepared using saccharin sodium salt hydrate (Sigma Aldrich, St. Louis, MI) and tap water. Sucrose solution (1.5% training, 5% *w/v* maintenance and testing) was prepared using sucrose (Sigma Aldrich, St. Louis, MI) and tap water. PACAP(6–38) was purchased from the American Peptide Company (Sunnyvale, CA). The peptide was dissolved in sterile isotonic saline in the presence of 1% bovine serum albumin (Sigma Aldrich, St. Louis, MI). A PAC1R short-hairpin knockdown adeno-associated virus (AAV1-CAG-*rADCYAP1R1*-shRNAmir-GFP, Vigene Biosciences, Rockville, MD, "AAV-PAC1R-KD"), and a control virus (AAV1-CAG-GFP, Addgene, Watertown, MA, "AAV-CTRL") were used for the NAcc Core *Adcyap1r1* gene (i.e. PAC1R) knockdown experiment.

2.3. Self-administration procedure: fixed ratio (FR)-1 schedule of reinforcement

Scr:sP and Wistar rats (n = 8/genotype for i.c.v. microinfusions; separate cohort of n = 7/brain area Scr:sP rats for intra-NAcc microinfusions) were trained to self-administer 10% *w/v* ethanol, as previously reported (Sabino et al., 2011), under a continuous FR1 schedule of reinforcement, wherein each response resulted in the delivery of 0.1 ml of liquid. Training culminated in daily ethanol self-administration sessions that were 30 min in duration as previously described (Sabino et al., 2006). During all sessions, rats were also allowed to press for water (0.1 ml) on the alternative lever. Drug testing began after approximately 6 weeks of daily 30 min alcohol FR1 sessions. For saccharin self-administration, Scr:sP rats (n = 6) were trained to self-administer a saccharin solution (0.02% *w/v*) under an FR1 schedule during 30 min sessions (Sabino et al., 2009). Drug testing began after approximately 4 weeks of daily sessions.

For sucrose self-administration, Scr:sP rats (n = 9) were trained under an FR1 schedule as above, first with 1.5% *w/v* sucrose, then with 5% *w/v* sucrose until stabilization. These concentrations of saccharin and sucrose were such to maintain response rates equivalent to those elicited by 10% *w/v* ethanol (see Results section) (see Supplementary Material for additional details).

2.4. Self-administration procedure: progressive ratio (PR) schedule of reinforcement

Scr:sP and Wistar rats (n = 11–12/genotype) were trained to self-administer 10% *w/v* ethanol under a Progressive Ratio (PR) schedule of reinforcement. Under this schedule, the number of responses required to produce one delivery of ethanol (10% *w/v*) increased with successive deliveries as in (Sabino et al., 2011). Sessions ended when subjects had not completed a ratio for 14 min or after 2 h, as we and others have previously reported (Gilpin and Koob, 2010; Sabino et al., 2011; Walker and Koob, 2007), with the last completed ratio defined as the breakpoint. Responses on the inactive lever were also recorded. Drug testing began after approximately 4 weeks of daily PR sessions (see Supplementary Material for additional details).

2.5. Alcohol-seeking procedure: second-order schedule of reinforcement

Scr:sP rats (n = 10) were trained under a second-order schedule of alcohol reinforcement adopted and modified from (Giuliano et al., 2012). Subjects were first trained to press a lever to acquire 0.05 ml of 10% *w/v* ethanol solution under a fixed ratio 1 (FR1) schedule of reinforcement; lever presses on the active lever resulted in ethanol solution delivery and illumination of a conditioned stimulus (CS) light above the active lever for 20 s, followed by lever retraction. The session ended when subjects reached 30 rewards or after 2 h, whichever occurred first. Subsequently, a fixed interval (FI) schedule of reinforcement was introduced, the interval increasing daily from FI1 min, to FI2, FI4, FI8, and FI10 min before stabilizing at FI15 min. After timing out of the FI, a single press on the active lever resulted in solution delivery. The volume of solution delivered after the FI progressively increased from 0.05 to 0.12 ml according to the FI schedule, and the session ended when subjects received 30 reinforcers or after 2 h. Finally, subjects were moved to a second-order schedule comprised of two intervals. During the FI15min, every 10th active lever press resulted in a brief CS presentation for 1 s above the active lever (FI15(FR10:S)); after the FI15 min, the 10th active lever press resulted in their first delivery of 0.12 ml of ethanol solution and CS presentation for 20 s. The session ended when subjects completed a second interval (after alcohol had been delivered) or after 40 min, whichever occurred first. Drug testing began after approximately 3 weeks of daily second-order schedule sessions (see Supplementary Material for additional details).

2.6. Intracranial surgeries

The surgical procedures were performed stereotaxically, as previously described (Dore et al., 2013; Iemolo et al., 2012). For microinfusion studies, rats underwent unilateral (for intracerebroventricular (i.c.v.) experiments) or bilateral (for NAcc Core and NAcc Shell) implantation of 24-gauge stainless steel cannulas (Plastics One, Roanoke, VA) under stereotaxic control (Kopf Instruments, Tujunga, CA), using the following coordinates (mm from bregma, DV from skull): i. c.v.: AP: − 1.0, ML: ±1.5, DV: − 2.3. NAcc Core: AP: +1.4, ML: ±2.4 (6-degree angle), DV: − 5.6; NAcc Shell: AP: +1.06, ML: ±0.75, DV: − 5.7. For the NAcc Core PAC1R knockdown study, AAV-PAC1R-KD or AAV-CTRL viral constructs were infused via a 22-gauge Hamilton syringe bilaterally into the NAcc Core, using the following coordinates (mm from bregma, DV from dura): AP: +1.4, ML: +2.5 (10° angle), DV: − 5.8. A total volume of 0.5 μl per side was used. The incisor bar was set at − 3.3 mm from the interaural line (flat skull) for all surgeries, according to Paxinos (Paxinos and Watson, 2007). Only data from animals with correct viral/cannula placement were included in the data analysis.

2.7. Cannula microinfusion procedures

Drug testing began when daily lever pressing performance in each schedule of reinforcement stabilized (<20% variation across three consecutive sessions). PACAP(6–38) (i.c.v.: 0, 3.75, 7.5 μg/rat in 5 μl/rat, intra-NAcc: 0, 0.5, 1.25 μg/side in 0.5 μl/side) was administered 30 min before the session; all experiments followed a Latin square within-subject design and at least two treatment-free days were allowed between treatment days (doses were chosen based on

previous reports (Burgos et al., 2013; Ferragud et al., 2021; Gargiulo et al., 2020b; Nguyen et al., 2020; Seiglie et al., 2019)).

2.8. NAcc core AAV-shRNA PAC1R knockdown

A cohort of Scr:sP rats (n = 17) was trained to self-administer alcohol in an FR1 schedule as described above. Once stable performance during the 30 min self-administration sessions was reached (<20% variation across three consecutive sessions), rats were matched based on baseline ethanol intake and body weight into two groups. Either AAV-PAC1R or AAV-CTRL virus was infused bilaterally into the NAcc Core as described above. Daily self-administration sessions were resumed 3 weeks after intracranial infusion, to allow for viral expression.

At the end of the experiment, rats were euthanized, and brains collected as described above. Immunohistochemistry for PAC1R and GFP in the NAcc Core was performed. Unbiased stereological counts by an experimenter blind to treatment groups were performed on a subset of rats (n = 6/group) as previously described (Ferragud et al., 2021) (see Supplementary Material for additional details).

2.9. PAC1R immunohistochemistry

Subjects of these experiments were two cohorts of Scr:sP and Wistar rats, ethanol naïve and ethanol experienced (n = 7/group and 4–5/group, respectively). Resulting ethanol intake in the ethanol experienced group were comparable to those of the pharmacological and viral experiments (see Results section). The ethanol-naïve cohort was never exposed to ethanol, while the ethanol-experienced cohort was trained to self-administer 10% *w/v* ethanol daily, first with home-cage access as described above, and then under an FR1 schedule of reinforcement for 4 weeks in 30 min sessions as described above and subsequently euthanized 1 h after the last operant session. Rats were perfused transcardially with phosphate-buffered saline and 4% paraformaldehyde (PFA); coronal brain sections, cut at 40 μm and including the NAcc Core and Shell (range: +2.52/+1.08 mm from bregma, every 6th section), were used for immunohistochemistry (IHC) for PAC1R by incubating them in anti-PAC1R primary antibody for 48 h at 4 °C and then in secondary antibody for 2 h at room temperature. PAC1R-labeled cell bodies were then counted by an experimenter blind to groups, after areas were outlined according to the Paxinos rat brain atlas (40) (see Supplementary Material for additional details).

2.10. Statistical analysis

Data from i.c.v. administration of PACAP(6–38) in FR1 and progressive ratio schedule of reinforcement were analyzed using two-way analyses of variance (ANOVAs), with Genotype as a between-subjects factor and Dose as a within-subject factor. Data from the saccharin and sucrose intake, and site-specific administration of PACAP(6–38) in FR1 (Scr:sP only) were analyzed using one-way ANOVAs, with Dose as a within-subject factor. Data from the second-order schedule of reinforcement were analyzed using two-way ANOVAs with Interval and Dose as a within-subject factors. Data from self-administration following AAV-shRNA PAC1R knockdown were analyzed using two-way ANOVAs with Virus as a between-subjects factor and Day as a within-subject factor. Data not normally

distributed were analyzed using the non-parametric Friedman's test. Pairwise post-hoc comparisons were made using either the Dunnett's (one-way ANOVA) or the Duncan (two-way ANOVAs) test; Student's *t*-test was used when comparing two groups. Significance was set at $p \leq 0.05$. The software/graphic packages used were Systat 11.0, InStat 3.0, Statistica 7.0, and Graphpad Prism 9.2.

3. Results

3.1. I.c.v. PAC1R antagonist PACAP(6–38) blocks excessive ethanol self-administration in alcohol-preferring, but not in outbred rats

As shown in Fig. 1, Scr:sP rats responded excessively for ethanol compared to outbred Wistar rats under a FR1 schedule of reinforcement (Fig. 1A; Genotype: $F(1,13) = 4.95$, $p < 0.05$). I.c.v. administration of the PAC1R antagonist PACAP(6–38) selectively and dose-dependently reduced the higher level of ethanol self-administration in Scr:sP rats, but not in outbred Wistar rats (Fig. 1A; Genotype*Dose: $F(2,26) = 3.96$, $p < 0.05$; Dose: $F(2,26) = 5.51$, $p < 0.01$, see also Suppl. Fig 2A for lever press data); post hoc analysis revealed a significant effect of the highest dose (7.5 μg) in Scr:sP compared to vehicle condition (40% reduction). At the highest dose microinfused, PACAP(6–38) fully blocked the excessive ethanol intake of Scr:sP rats as compared to vehicle-treated Wistar rats ($p = 0.70$). As shown in Fig. 1B, responding for water was not reliably affected by PACAP(6–38) in either genotype (Genotype*-Dose: $F(2,26) = 0.23$, n. s.; Dose: $F(2,26) = 0.44$, n. s., see Suppl Fig. 2B for lever press data).

3.2. I.c.v. PAC1R antagonist PACAP(6–38) reduces the motivation to drink ethanol in progressive ratio in alcohol-preferring, but not in outbred rats

Scr:sP rats showed increased breakpoint and total number of responses for ethanol compared to outbred Wistar rats under a progressive ratio schedule of reinforcement (breakpoint: Fig. 1C; Genotype: $F(1,21) = 17.4$, $p < 0.001$). I. c.v. administration of the PAC1R antagonist PACAP(6–38) decreased the breakpoint for ethanol in Scr:sP rats, but not in outbred Wistar rats (Fig. 1C; Genotype*Dose: $F(2,42) = 3.91$, $p < 0.05$; Dose: $F(2,42) = 1.97$, n. s.). PACAP(6–38) also reduced the total responses for ethanol on the active lever selectively in Scr:sP rats (Fig. 1D; Genotype*Dose: $F(2,42) = 4.49$, $p < 0.05$; Dose: $F(2,42) = 2.34$, n. s.). Post-hoc analysis showed that the highest dose of PACAP (6–38) significantly reduced the breakpoint (25% of reduction vs. vehicle condition) and the number of active lever presses (32% of reduction vs. vehicle condition) in Scr:sP rats. The antagonist treatment had no effect on the number of inactive lever presses (Fig. 1E; Genotype*Dose: $F(2,42) = 0.42$, n. s.; Dose: $F(2,42) = 1.30$, n. s.).

3.3. I.c.v. PAC1R antagonist PACAP(6–38) decreases alcohol-seeking behavior in alcohol-preferring rats

As shown in Fig. 2A, PACAP(6–38) decreased alcohol-seeking behavior in Scr:sP rats in both intervals, i.e. both before and after alcohol delivery (Interval: $F(1,9) = 7.04$, $p < 0.05$; Dose: $F(2,18) = 4.69$, $p < 0.05$) of a second-order schedule of reinforcement, in which alcohol-seeking behavior is maintained by both the self-administered alcohol and the response-contingent presentation of an alcohol-associated CS. The highest dose of PACAP

(6–38) significantly decreased the number of lever presses during both the first pre-ingestive first interval (43% reduction, Fig. 2A) and the post-ingestive second interval (57% reduction, Fig. 2A). The treatment had no effect on inactive lever presses in either interval (Interval: $F(1,9) = 20.41$, n. s.; Dose: $F(2,18) = 1.19$, n. s.; Fig. 2B).

3.4. I.c.v. PAC1R antagonist PACAP(6–38) has no effect on saccharin or sucrose intake in alcohol-preferring rats

As shown in Fig. 2C, ic v. administration of the PAC1R antagonist PACAP(6–38) did not affect lever pressing for saccharin solution in Scr: sP rats in an FR1 schedule of reinforcement ($F(2,10) = 0.13$, n. s.; lever press data in Fig. 3A). As desired, the concentration of saccharin chosen (0.02% *w/v*) maintained levels of responding under vehicle conditions that matched those obtained with ethanol (37.3 ± 4.3 and 39.3 ± 7.9 average of lever presses for ethanol and saccharin, respectively). PACAP (6–38) had no effect on concurrent water intake ($F(2,10) = 0.71$, n. s.) (data not shown, Veh: 0.28 ± 0.20 ml/kg, 3.75 mg/kg dose: 0.12 ± 0.08 ml/kg, 7.5 mg/kg dose: 0.40 ± 0.23 ml/kg).

I.c.v. administration of the PAC1R antagonist PACAP(6–38) in Scr:sP rats did not affect sucrose self-administration under an FR1 schedule of reinforcement (Fig. 2D; $F(2,16) = 0.52$, n. s.; lever press data in Fig. 3B). The sucrose concentration (5% *w/v*) was chosen so that the caloric intake matched that of the ethanol solution (10% *w/v*) in the ethanol self-administration experiment (2.61 ± 0.30 and 2.34 ± 0.14 average kcal per session for ethanol and sucrose, respectively). PACAP(6–38) had no effect on concurrent water intake during sucrose testing ($F(2, 16) = 0.98$, n. s.) (data not shown, Veh: 0.31 ± 0.14 ml/kg, 3.75 mg/kg dose: 0.27 ± 0.10 ml/kg, 7.5 mg/kg dose: 0.11 ± 0.06 ml/kg).

3.5. PAC1R levels are increased in NAcc core of alcohol-preferring rats following alcohol exposure

No differences in PAC_1R immunoreactivity (PAC1R positive cells) were observed between alcohol naïve Scr:sP rats and alcohol naïve Wistar rats in the NAcc Core ($t(12) = 0.94$, *n. s.*, Fig. 3A), or in the NAcc Shell ($t(12) = 0.60$, *n. s.*, Fig. 3B). Interestingly, following exposure to alcohol, as shown in Fig. 3A, significantly higher levels of PAC1R-labeled cells were observed in the NAcc Core of ethanol-experienced Scr:sP rats compared to Wistars ($t(7) = 2.94$, $p < 0.05$). Again, no significant differences in PAC1R levels were observed in the NAcc Shell ($t(7) = 2.36$, n. s.) (Fig. 3B). Since the two experiments assessing PAC1R levels in the cohorts of alcohol naïve vs. alcohol experienced rats were performed independently at different times, we were unfortunately unable to directly compare the effects of alcohol exposure on PAC1R in each genotype.

3.6. PAC1R antagonist PACAP(6–38) microinfusion in the NAcc core blocks excessive alcohol drinking in alcohol-preferring rats

Microinfusions of the PAC1R antagonist PACAP(6–38) into the NAcc Core significantly decreased excessive ethanol intake in Scr:sP rats, as shown in Fig. 4A ($F(2,12) = 25.73$, $p < 0.001$; lever press data in Suppl. Fig. 4A). Post hoc analysis revealed that both doses of PACAP (6–38) significantly reduced ethanol intake (0.5 μg/side: 30%, 1.25 μg/side: 41%, compared to vehicle). In contrast, no effects were observed when PACAP(6–38) was

microinfused into the NAcc Shell, as shown in Fig. 4C ($F(2,12) = 1.37$, n. s.; lever press data in Suppl. Fig. 4C), supporting the immunohistochemical findings that the NAcc Core PAC1Rs are those involved in the control of excessive alcohol drinking in Scr:sP rats (see Sup. Fig. 1 for cannula placements). Water intake was not affected by PACAP(6–38) microinfusion in either subregion of the NAcc (non-parametric data, Core: $\chi^2(2, N = 7) = 0.50$, n. s.; Shell: $\chi^2(2, N = 7) = 1.68$ n. s., Fig. 4B and D; lever press data in Suppl. Fig. 4B and 4D).

3.7. Virally-mediated PAC1R short-hairpin RNA knockdown in the NAcc core decreases excessive alcohol drinking in alcohol-preferring rats

Rats bilaterally infused with a PAC1R short-hairpin RNA knockdown virus into the NAcc Core (AAV-PAC1R-KD) drank significantly less alcohol than rats injected with an AAV-CTRL control virus (Virus: $F(1,16) = 4.76$, $p < 0.05$, Fig. 5A, and representative image in Fig. 5C; lever press data in Suppl. Fig. 5A), further supporting the site-specificity of this effect. This effect became more pronounced over time, with the AAV-PAC1R-KD rats drinking 25% less than AAV-CTRL in the last 7 sessions (AAV-CTRL, Mean ± SEM: 1.41 ± 0.10 g/kg; AAV-PAC1R-KD, Mean ± SEM: 1.06 ± 0.08 g/kg). PAC1R knockdown in the NAcc core had no significant effect on water intake (Virus: $F(1,16) = 0.33$, n. s., Fig. 5B; lever press data in Suppl. Fig. 5B). AAV-PAC1R-KD rats expressed 43.3% fewer PAC1R + cells than AAV-CTRL rats; indeed, as expected, the AAV-PAC1R-KD significantly reduced the amount of PAC1R immunoreactivity in the NAcc Core ($t(10) = 3.19$, $p < 0.01$, Fig. 5D and E), but not in the NAcc Shell ($t(10) = -1.58$, n. s., not shown).

4. Discussion

The neuropeptide PACAP has recently been proposed to play a role in the regulation of alcohol drinking. This series of studies showed that: 1) i. c.v. administration of the PAC1R antagonist PACAP(6–38) decreased excessive alcohol drinking, motivation to drink, and cue-maintained alcohol seeking in Scr:sP rats; 2) i. c.v. PACAP(6–38) did not affect responding for ethanol or water in outbred Wistar rats; 3) ethanol-exposed Scr:sP rats had higher levels of PAC1R in the NAcc Core, compared to outbred Wistar rats; 4) both pharmacological antagonism and viral vector-mediated shRNA knockdown of PAC1R specifically in the NAcc Core reduced alcohol drinking in Scr:sP rats. Overall, our data strongly implicate the NAcc Core PACAP/PAC1R system in regulating excessive ethanol drinking.

Administration of the PAC1R antagonist PACAP(6–38) i.c.v. dose-dependently and selectively decreased alcohol drinking in alcohol preferring Scr:sP rats. I.c.v. PACAP(6–38) not only blocked excessive drinking in Scr:sP rats, bringing intake levels back to those of control outbred rats, but also reduced the breakpoint under a progressive ratio schedule of reinforcement, a measure of the motivation to obtain alcohol in which the influence of local response rates on performance are reduced. Importantly, PAC1R blockade reduced alcohol-seeking behavior measured in rats responding under a second-order schedule of reinforcement (Blasio et al., 2015; Domi et al., 2021; Giuliano et al., 2015; Lamb et al., 2015). This procedure, established initially with psychostimulant and opiate reinforcers

(Giuliano et al., 2012; Ito et al., 2004), is characterized by the maintenance of responding by the contingent presentation of drug-paired stimuli acting as conditioned reinforcers prior to eventual delivery of drug (Everitt and Robbins, 2000). PACAP(6–38) dose-dependently reduced the number of active lever presses in the second-order schedule and, in particular, during the first interval of the session, which is unaffected by the pharmacological effects of recently administered alcohol, suggesting an effect of PACAP (6–38) on alcohol seeking responses maintained by contingently presented alcohol-associated conditioned stimuli (Everitt and Robbins, 2000; Giuliano et al., 2012). Since the PAC1R antagonist PACAP(6–38) is not a small molecule but is instead a peptide, it is therefore unsuitable for systemic administration, and was thus given i.c.v.

The observation that the PAC1R antagonist PACAP(6–38) did not reduce ethanol self-administration in outbred control Wistar rats, did not reduce self-administration of water, and did not reduce intake of either a non-caloric (saccharin) or a caloric (sucrose) alternative reinforcer shows that PACAP(6–38) does not produce any malaise-like or other non-specific behavior. It also rules out that PAC1R antagonism reduces caloric intake, in line with the established anorectic effect of PAC1R *agonism* (Dore et al., 2013; Hurley et al., 2016, 2019; Iemolo et al., 2015; Krashes et al., 2014). The lack of effect of the antagonist on basal levels of alcohol drinking in Wistar rats suggests that its effects are selective towards high levels of drinking. Scr:sP rats descend from Sardinian alcohol preferring rats, which were selected from outbred Wistar rats to prefer 10% alcohol and represent an established model for AUD, as they voluntarily drink high volumes of ethanol, have a heritable component, and show predictive validity for AUD (Bohman et al., 1981; Cloninger et al., 1981; Colombo, 1997; Colombo et al., 2006; McBride and Li, 1998; Prescott and Kendler, 1999; Sigvardsson et al., 1996). Wistar rats were used as a control group because Scr:sP (and sP) rats were originally selected from Wistar rats, and also because using non-preferring rats might raise concerns about whether observed differences are associated with excessive drinking as opposed to ethanol aversion, which is associated with very distinct molecular changes (Economidou et al., 2008; Hansson et al., 2007; Saba et al., 2001; Sabino et al., 2009). Overall, the specificity of drug treatment effects indicates that this system is recruited and plays a functional role in Scr:sP rats when the brain reward system is activated by alcohol, or the anticipation of alcohol, while it plays a limited role under baseline conditions.

The observed selective effect of the PAC1R antagonist in alcohol-preferring Scr:sP rats led us to hypothesize that alterations in PAC1R levels could be involved in the excessive drinking phenotype observed. Using immunohistochemistry, we found no difference in the levels of PAC1R positive cells in the NAcc Core or NAcc Shell of Scr:sP rats, compared to outbred Wistar rats, prior to any ethanol exposure. We found significantly higher levels (188.3 ± 20.2% increase) of PAC1R in the NAcc Core of ethanol experienced Scr:sP rats, compared to Wistars, following 4 weeks of ethanol self-administration. Since the two experiments assessing PAC1R levels in the cohorts of alcohol naïve vs. alcohol experienced rats were performed independently, we were unable to directly compare the effects of alcohol exposure on PAC1R in each of the genotypes, and we recognize this is a significant limitation of the current study because of possible effects of environmental conditions on the analysis. Since Scr:sP rats consume more ethanol than Wistars, and considering that the two genotypes did not differ in NAcc Core PAC1R levels prior to ethanol exposure, it is likely

that differences in PAC1R levels are a result of the greater alcohol consumption (or of a gene by environment interaction) in Scr:sP rats, which may upregulate PAC1R. This, in turn, may underlie the increased sensitivity of drinking to the PAC1R blockade. It cannot be ruled out, however, than the differences observed between genotypes are instead a compensatory mechanism to counteract a different pathway upregulated by alcohol intake in alcohol-preferring rats, such as the Map- or Cam-kinase pathways (Hansson et al., 2008; Rosas et al., 2014; Sommer et al., 2006). Even though it is considered a semi-quantitative technique (Cregger et al., 2006; Taylor and Levenson, 2006), immunohistochemistry was used in this study, instead of a technique requiring tissue punches, as it allows analysis of any differences with regional specificity, which is important as adjacent brain regions frequently play different, or even opposite, roles in behavior. Collectively, these immunohistochemical findings support the antagonist action of PACAP(6–38) at PAC1R, rather than VPAC receptors (Ferragud et al., 2021; Seiglie et al., 2019) and point to the NAcc Core as a key site at which PAC1R activation may drive high levels of alcohol drinking. PACAP(6–38) has been reported to also act as an agonist of MrgB3 receptors (Pedersen et al., 2019); however, the observation that, in the NAcc Core, PAC1R levels are higher in alcohol-preferring rats and that PAC1R knockdown also reduces ethanol intake strongly discount the potential alternative interpretation that the effects of PACAP(6–38) are mediated by the activation of MrgB3 receptors (Pedersen et al., 2019).

A relationship between alcohol and PACAP had been suggested by Moore and colleagues who showed increased sensitivity to alcohol in the *Drosophila* mutant *cheapdate*, which carries a mutation of a PACAP-like gene (Moore et al., 1998). Furthermore, in a series of previous investigations, whole body PACAP knockout mice had been shown to display reduced sensitivity to some effects of alcohol, but higher preference for it (Tanaka et al., 2004, 2010). This study did not report direct differences in alcohol intake between wildtype and knockout animals (Tanaka et al., 2010). It is conceivable that the higher ethanol preference in these mice could be due to the reduced sensitivity to its effects, which would be parsimonious with our finding that reducing PACAP action at the PAC1R reduces alcohol intake/effects. However, direct comparisons across species and in home-cage access vs. operant conditions is always difficult, and in this case possible compensatory mechanisms occurring in whole-body knockout animals should also be considered.

We recently reported an increase in self-administration of alcohol, and motivation to drink specifically in Scr:sP rats following long-term PAC1R knockdown in the NAcc Shell (Minnig et al., 2021), which supports a role for this system in the NAcc Shell to decrease alcohol drinking, perhaps involving upstream afferents from the infralimbic cortex (Koob and Volkow, 2016b; LaLumiere et al., 2012). In the present study, however, acute pharmacological antagonism of PAC1R in NAcc Shell did not affect ethanol self-administration; this discrepancy with the previous findings could be due to the chronic vs. acute manipulation, i.e. that acute pharmacological antagonism of PAC1R in the Shell in this study could be insufficient to reverse plastic changes which may occur downstream of PAC1R. Interestingly, in this study, we also saw no differences in the number of PAC1R-positive cell numbers in the NAcc Shell between Scr:sP and Wistar rats either before or after ethanol exposure. This result doesn’t rule out that removing the NAcc Shell PAC1R “brake” via its long-term downregulation (i.e. knockdown) cannot affect drinking. More

studies will be needed to better understand the intricacies of these circuits in the context of the PACAP/PAC1R system.

A recent report also concluded that administration of the PAC1R antagonist PACAP(6–38) in the NAcc Shell had no effect on alcohol drinking, but microinfusion of the neuropeptide itself, PACAP-38, into the NAcc Core decreased alcohol intake 30 min later in Long-Evans rats exposed to 4 weeks of ethanol via home cage two-bottle choice (Gargiulo et al., 2020b). This finding is in apparent contrast with our data showing no change in alcohol self-administration following antagonism of PAC1R in another outbred strain, Wistar rats. A possible explanation for this discrepancy is that the length and method of ethanol exposure were different in the two studies (e.g. home cage vs. operant, 24 h a day vs. 30 min), or simply strain differences (Long Evans vs. Wistars). In alcohol-preferring rats specifically, we observe decreased alcohol self-administration following antagonism of PAC1R, as well as decreased self-administration following short-hairpin RNA knockdown of PAC1R in the NAcc Core. The disagreement between our finding here and those expected based off on experiments by Gargiulo et al. may be due to the fact our studies were performed using alcohol-preferring rats, which reinforces that genetic predisposition vs. environmental exposure to alcohol may differentially alter the system.

One limitation of our study is that only male animals were used; even though no sex differences have been reported in self-administration of alcohol in the specific line of alcohol-preferring rats used here (Lorrai et al., 2019), further investigations of sex differences in the PACAP/PAC1R system in alcohol drinking are warranted, especially in light of sex-specific findings in models of other neuropsychiatric disorders (Kirry et al., 2018; Nega et al., 2020; Rajbhandari et al., 2021; Ramikie and Ressler, 2016; Ressler et al., 2011; Ross et al., 2020), as well as in human studies (Dragan et al., 2017; Kovanen et al., 2010).

In conclusion, our behavioral and molecular findings provide evidence that PAC1R in the NAcc Core plays a key role in the vulnerability to drink high levels of alcohol. This research identifies the PACAP/PAC1R system as a promising therapeutic target for the treatment of AUD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Tayun Park, Maria Echeveste Sanchez, Kayhan Kazbay, Lauren Lepeak, and Sophia Miracle for the technical assistance. We also thank The Scripps Research Institute and the University of Cagliari, CNR Neuroscience Institute, for providing the Scr:sP and the original sP rats, respectively.

Funding and disclosure

This publication was made possible thanks to grant numbers AA024439 (VS), AA025038 (VS), AA026051 (PC), and AA028184 (MAM), all from the National Institute on Alcohol Abuse and Alcoholism (NIAAA), and the Boston University's Undergraduate Research Opportunities Program (UROP). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health. The authors declare no conflict of interest and no biomedical financial interests.

References

Agarwal A, Halvorson LM, Legradi G, 2005. Pituitary adenylate cyclase-activating polypeptide (PACAP) mimics neuroendocrine and behavioral manifestations of stress: evidence for PKA-mediated expression of the corticotropin-releasing hormone (CRH) gene. Brain Res. Mol. Brain Res. 138, 45–57. [PubMed: 15882914]

Association AP, 2013. Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Publishing, Arlington, VA.

Bell RL, Lopez MF, Cui C, Egli M, Johnson KW, Franklin KM, Becker HC, 2013. Ibudilast reduces alcohol drinking in multiple animal models of alcohol dependence. Addiction Biol.

Blasio A, Valenza M, Iyer MR, Rice KC, Steardo L, Hayashi T, Cottone P, Sabino V, 2015. Sigma-1 receptor mediates acquisition of alcohol drinking and seeking behavior in alcohol-preferring rats. Behav. Brain Res. 287, 315–322. [PubMed: 25848705]

Bohman M, Sigvardsson S, Cloninger CR, 1981. Maternal inheritance of alcohol abuse. Cross-fostering analysis of adopted women. Arch. Gen. Psychiatr. 38, 965–969. [PubMed: 7283667]

Burgos JR, Iresjo BM, Smedh U, 2013. Pituitary adenylate cyclase-activating polypeptide 6–38 blocks cocaine- and amphetamine-regulated transcript Peptide-induced hypophagia in rats. PLoS One 8, e72347. [PubMed: 23967296]

Ciccocioppo R, Hyytia P, 2006. The genetic of alcoholism: learning from 50 years of research. Addiction Biol. 11, 193–194.

Cloninger CR, Bohman M, Sigvardsson S, 1981. Inheritance of alcohol abuse. Cross-fostering analysis of adopted men. Arch. Gen. Psychiatr. 38, 861–868. [PubMed: 7259422]

Colombo G, 1997. ESBRA-Nordmann 1996 Award Lecture: ethanol drinking behaviour in Sardinian alcohol-preferring rats. Alcohol Alcohol 32, 443–453. [PubMed: 9269852]

Colombo G, Lobina C, Carai MA, Gessa GL, 2006. Phenotypic characterization of genetically selected Sardinian alcohol-preferring (sP) and -non-preferring (sNP) rats. Addiction Biol. 11, 324–338.

Corbit LH, Fischbach SC, Janak PH, 2016. Nucleus accumbens core and shell are differentially involved in general and outcome-specific forms of Pavlovian-instrumental transfer with alcohol and sucrose rewards. Eur. J. Neurosci. 43, 1229–1236. [PubMed: 26970240]

Corbit LH, Muir JL, Balleine BW, 2001. The role of the nucleus accumbens in instrumental conditioning: evidence of a functional dissociation between accumbens core and shell. J. Neurosci. 21, 3251–3260. [PubMed: 11312310]

Crabbe JC, 2014. Rodent models of genetic contributions to motivation to abuse alcohol. Nebr. Symp. Motiv. 61, 5–29. [PubMed: 25306777]

Cregger M, Berger AJ, Rimm DL, 2006. Immunohistochemistry and quantitative analysis of protein expression. Arch. Pathol. Lab Med. 130, 1026–1030. [PubMed: 16831029]

Domi E, Domi A, Adermark L, Heilig M, Augier E, 2021. Neurobiology of alcohol seeking behavior. J. Neurochem.

Dore R, Iemolo A, Smith KL, Wang X, Cottone P, Sabino V, 2013. CRF mediates the anxiogenic and anti-rewarding, but not the anorectic effects of PACAP. Neuropsychopharmacology 38, 2160–2169. [PubMed: 23657440]

Dragan WL, Czerski PM, Dragan M, 2017. PAC1 receptor (ADCYAP1R1) genotype and problematic alcohol use in a sample of young women. Neuropsychiatric Dis. Treat 13, 1483–1489.

Economidou D, Hansson AC, Weiss F, Terasmaa A, Sommer WH, Cippitelli A, Fedeli A, Martin-Fardon R, Massi M, Ciccocioppo R, Heilig M, 2008. Dysregulation of nociceptin/orphanin FQ activity in the amygdala is linked to excessive alcohol drinking in the rat. Biol. Psychiatr. 64, 211–218.

Everitt BJ, Robbins TW, 2000. Second-order schedules of drug reinforcement in rats and monkeys: measurement of reinforcing efficacy and drug-seeking behaviour. Psychopharmacology (Berl) 153, 17–30. [PubMed: 11255926]

Feany MB, Quinn WG, 1995. A neuropeptide gene defined by the Drosophila memory mutant amnesiac. Science 268, 869–873. [PubMed: 7754370]

Feduccia AA, Simms JA, Mill D, Yi HY, Bartlett SE, 2014. Varenicline decreases ethanol intake and increases dopamine release via neuronal nicotinic acetylcholine receptors in the nucleus accumbens. Br. J. Pharmacol. 171, 3420–3431. [PubMed: 24628360]

Ferragud A, Velazquez-Sanchez C, Minnig MA, Sabino V, Cottone P, 2021. Pituitary adenylate cyclase-activating polypeptide (PACAP) modulates dependence-induced alcohol drinking and anxiety-like behavior in male rats. Neuropsychopharmacology 46, 509–518. [PubMed: 33191400]

Gargiulo AT, Curtis GR, Barson JR, 2020a. Pleiotropic pituitary adenylate cyclase-activating polypeptide (PACAP): novel insights into the role of PACAP in eating and drug intake. Brain Res. 1729, 146626. [PubMed: 31883848]

Gargiulo AT, Pirino BE, Curtis GR, Barson JR, 2020b. Effects of pituitary adenylate cyclase-activating polypeptide isoforms in nucleus accumbens subregions on ethanol drinking. Addiction Biol, e12972

Gessa GL, 2016. The long pursued Holy Grail of the true “alcoholic” rat. Brain Res. 1645, 55–57. [PubMed: 26867703]

Ghatei MA, Takahashi K, Suzuki Y, Gardiner J, Jones PM, Bloom SR, 1993. Distribution, molecular characterization of pituitary adenylate cyclase-activating polypeptide and its precursor encoding messenger RNA in human and rat tissues. J. Endocrinol. 136, 159–166. [PubMed: 8094091]

Gilpin NW, Koob GF, 2010. Effects of beta-adrenoceptor antagonists on alcohol drinking by alcohol-dependent rats. Psychopharmacology (Berl) 212, 431–439. [PubMed: 20676608]

Giuliano C, Goodlett CR, Economidou D, García-Pardo MP, Belin D, Robbins TW, Bullmore ET, Everitt BJ, 2015. The novel μ-opioid receptor antagonist GSK1521498 decreases both alcohol seeking and drinking: evidence from a new preclinical model of alcohol seeking. Neuropsychopharmacology 40, 2981–2992. [PubMed: 26044906]

Giuliano C, Robbins TW, Nathan PJ, Bullmore ET, Everitt BJ, 2012. Inhibition of opioid transmission at the mu-opioid receptor prevents both food seeking and binge-like eating. Neuropsychopharmacology 37, 2643–2652. [PubMed: 22805601]

Goldman D, Oroszi G, Ducci F, 2005. The genetics of addictions: uncovering the genes. Nat. Rev. Genet. 6, 521–532. [PubMed: 15995696]

Gray SL, Yamaguchi N, Vencova P, Sherwood NM, 2002. Temperature-sensitivé phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. Endocrinology 143, 3946–3954. [PubMed: 12239106]

Hammack SE, Cheung J, Rhodes KM, Schutz KC, Falls WA, Braas KM, May V, 2009. Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): roles for PACAP in anxiety-like behavior. Psychoneuroendocrinology 34, 833–843. [PubMed: 19181454]

Hammack SE, Roman CW, Lezak KR, Kocho-Shellenberg M, Grimmig B, Falls WA, Braas K, May V, 2010. Roles for pituitary adenylate cyclase-activating peptide (PACAP) expression and signaling in the bed nucleus of the stria terminalis (BNST) in mediating the behavioral consequences of chronic stress. J. Mol. Neurosci. 42, 327–340. [PubMed: 20405238]

Hansson AC, Cippitelli A, Sommer WH, Ciccocioppo R, Heilig M, 2007. Region-specific down-regulation of Crhr1 gene expression in alcohol-preferring msP rats following ad lib access to alcohol. Addiction Biol. 12, 30–34.

Hansson AC, Rimondini R, Neznanova O, Sommer WH, Heilig M, 2008. Neuroplasticity in brain reward circuitry following a history of ethanol dependence. Eur. J. Neurosci. 27, 1912–1922. [PubMed: 18412612]

Harmar AJ, Fahrenkrug J, Gozes I, Laburthe M, May V, Pisegna JR, Vaudry D, Vaudry H, Waschek JA, Said SI, 2012. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR review 1. Br. J. Pharmacol. 166, 4–17. [PubMed: 22289055]

Hawke Z, Ivanov TR, Bechtold DA, Dhillon H, Lowell BB, Luckman SM, 2009. PACAP neurons in the hypothalamic ventromedial nucleus are targets of central leptin signaling. J. Neurosci. 29, 14828–14835. [PubMed: 19940178]

He DY, Vagts AJ, Yaka R, Ron D, 2002. Ethanol induces gene expression via nuclear compartmentalization of receptor for activated C kinase 1. Mol. Pharmacol. 62, 272–280. [PubMed: 12130678]

Heilig M, Koob GF, 2007. A key role for corticotropin-releasing factor in alcohol dependence. Trends Neurosci. 30, 399–406. [PubMed: 17629579]

Hurley MM, Maunze B, Block ME, Frenkel MM, Reilly MJ, Kim E, Chen Y, Li Y, Baker DA, Liu QS, Choi S, 2016. Pituitary adenylate-cyclase activating polypeptide regulates hunger- and palatability-induced binge eating. Front. Neurosci. 10, 383. [PubMed: 27597817]

Hurley MM, Robble MR, Callan G, Choi S, Wheeler RA, 2019. Pituitary adenylate cyclase-activating polypeptide (PACAP) acts in the nucleus accumbens to reduce hedonic drive. Int. J. Obes. 43, 928–932.

Iemolo A, Ferragud A, Cottone P, Sabino V, 2015. Pituitary adenylate cyclase-activating peptide in the central amygdala causes anorexia and body weight loss via the melanocortin and the TrkB systems. Neuropsychopharmacology 40, 1846–1855. [PubMed: 25649277]

Iemolo A, Seiglie M, Blasio A, Cottone P, Sabino V, 2016. Pituitary adenylate cyclase-activating polypeptide (PACAP) in the central nucleus of the amygdala induces anxiety via melanocortin receptors. Psychopharmacology (Berl) 233, 3269–3277. [PubMed: 27376948]

Iemolo A, Valenza M, Tozier L, Knapp CM, Kornetsky C, Steardo L, Sabino V, Cottone P, 2012. Withdrawal from chronic, intermittent access to a highly palatable food induces depressive-like behavior in compulsive eating rats. Behav. Pharmacol. 23, 593–602. [PubMed: 22854309]

Inglott MA, Farnham MM, Pilowsky PM, 2011. Intrathecal PACAP-38 causes prolonged widespread sympathoexcitation via a spinally mediated mechanism and increases in basal metabolic rate in anesthetized rat. Am. J. Physiol. Heart Circ. Physiol. 300, H2300–H2307. [PubMed: 21460201]

Ito R, Robbins TW, Everitt BJ, 2004. Differential control over cocaine-seeking behavior by nucleus accumbens core and shell. Nat. Neurosci. 7, 389–397. [PubMed: 15034590]

Jiang SZ, Eiden LE, 2016. Activation of the HPA axis and depression of feeding behavior induced by restraint stress are separately regulated by PACAPergic neurotransmission in the mouse. Stress 19, 374–382. [PubMed: 27228140]

Jones JD, Comer SD, Kranzler HR, 2015. The pharmacogenetics of alcohol use disorder. Alcohol Clin. Exp. Res. 39, 391–402. [PubMed: 25703505]

Kirry AJ, Herbst MR, Poirier SE, Maskeri MM, Rothwell AC, Twining RC, Gilmartin MR, 2018. Pituitary adenylate cyclase-activating polypeptide (PACAP) signaling in the prefrontal cortex modulates cued fear learning, but not spatial working memory, in female rats. Neuropharmacology 133, 145–154. [PubMed: 29353055]

Koob GF, 2014. Neurocircuitry of alcohol addiction: synthesis from animal models. Handb. Clin. Neurol. 125, 33–54. [PubMed: 25307567]

Koob GF, Volkow ND, 2010. Neurocircuitry of addiction. Neuropsychopharmacology 35, 217–238. [PubMed: 19710631]

Koob GF, Volkow ND, 2016a. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatr. 3, 760–773.

Koob GF, Volkow ND, 2016b. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatr. 3, 760–773.

Kovanen L, Saarikoski ST, Haukka J, Pirkola S, Aromaa A, Lonnqvist J, Partonen T, 2010. Circadian clock gene polymorphisms in alcohol use disorders and alcohol consumption. Alcohol Alcohol 45, 303–311. [PubMed: 20554694]

Kranzler HR, Soyka M, 2018. Diagnosis and pharmacotherapy of alcohol use disorder: a review. JAMA 320, 815–824. [PubMed: 30167705]

Krashes MJ, Shah BP, Madara JC, Olson DP, Strochlic DE, Garfield AS, Vong L, Pei H, Watabe-Uchida M, Uchida N, Liberles SD, Lowell BB, 2014. An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. Nature 507, 238–242. [PubMed: 24487620]

LaLumiere RT, Smith KC, Kalivas PW, 2012. Neural circuit competition in cocaine-seeking: roles of the infralimbic cortex and nucleus accumbens shell. Eur. J. Neurosci. 35, 614–622. [PubMed: 22321070]

Lamb RJ, Pinkston JW, Ginsburg BC, 2015. Ethanol self-administration in mice under a second-order schedule. Alcohol 49, 561–570. [PubMed: 26254963]

Lorrai I, Contini A, Gessa GL, Mugnaini C, Corelli F, Colombo G, Maccioni P, 2019. Operant, oral alcohol self-administration: sex differences in Sardinian alcohol-preferring rats. Alcohol 79, 147–162. [PubMed: 31029630]

Malinen H, Hyytiä P, 2008. Ethanol self-administration is regulated by CB1 receptors in the nucleus accumbens and ventral tegmental area in alcohol-preferring AA rats. Alcohol Clin. Exp. Res. 32, 1976–1983. [PubMed: 18782338]

Masuo Y, Suzuki N, Matsumoto H, Tokito F, Matsumoto Y, Tsuda M, Fujino M, 1993. Regional distribution of pituitary adenylate cyclase activating polypeptide (PACAP) in the rat central nervous system as determined by sandwich-enzyme immunoassay. Brain Res. 602, 57–63. [PubMed: 8095427]

McBride WJ, Li TK, 1998. Animal models of alcoholism: neurobiology of high alcohol-drinking behavior in rodents. Crit. Rev. Neurobiol. 12, 339–369. [PubMed: 10348615]

McBride WJ, Rodd ZA, Bell RL, Lumeng L, Li TK, 2014. The alcohol-preferring (P) and high-alcohol-drinking (HAD) rats–animal models of alcoholism. Alcohol 48, 209–215. [PubMed: 24268381]

Miles OW, May V, Hammack SE, 2019. Pituitary adenylate cyclase-activating peptide (PACAP) signaling and the dark side of addiction. J. Mol. Neurosci. 68, 453–464. [PubMed: 30074172]

Minnig MA, Park T, Echeveste Sanchez M, Cottone P, Sabino V, 2021. Viral-mediated knockdown of nucleus accumbens shell PAC1 receptor promotes excessive alcohol drinking in alcohol-preferring rats. Front. Behav. Neurosci. 15, 787362. [PubMed: 34924973]

Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH, 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem. Biophys. Res. Commun. 164, 567–574. [PubMed: 2803320]

Moore MS, DeZazzo J, Luk AY, Tully T, Singh CM, Heberlein U, 1998. Ethanol intoxication in Drosophila: genetic and pharmacological evidence for regulation by the cAMP signaling pathway. Cell 93, 997–1007. [PubMed: 9635429]

Mounien L, Do Rego JC, Bizet P, Boutelet I, Gourcerol G, Fournier A, Brabet P, Costentin J, Vaudry H, Jegou S, 2009. Pituitary adenylate cyclase-activating polypeptide inhibits food intake in mice through activation of the hypothalamic melanocortin system. Neuropsychopharmacology 34, 424–435. [PubMed: 18536705]

Mustafa T, Jiang SZ, Eiden AM, Weihe E, Thistlethwaite I, Eiden LE, 2015. Impact of PACAP and PAC1 receptor deficiency on the neurochemical and behavioral effects of acute and chronic restraint stress in male C57BL/6 mice. Stress 18, 408–418. [PubMed: 25853791]

Nega S, Marquez P, Hamid A, Ahmad SM, Lutfy K, 2020. The role of pituitary adenylyl cyclase activating polypeptide in affective signs of nicotine withdrawal. J. Neurosci. Res.

Nguyen TT, Kambe Y, Kurihara T, Nakamachi T, Shintani N, Hashimoto H, Miyata A, 2020. Pituitary adenylate cyclase-activating polypeptide in the ventromedial hypothalamus is responsible for food intake behavior by modulating the expression of agouti-related peptide in mice. Mol. Neurobiol. 57, 2101–2114. [PubMed: 31927724]

Palkovits M, Somogyvari-Vigh A, Arimura A, 1995. Concentrations of pituitary adenylate cyclase activating polypeptide (PACAP) in human brain nuclei. Brain Res. 699, 116–120. [PubMed: 8616598]

Paxinos G, Watson C, 2007. The Rat Brain in Stereotaxic Coordinates. Academic Press/Elsevier, Amsterdam; Boston.

Pedersen SH, la Cour SH, Calloe K, Hauser F, Olesen J, Klaerke DA, Jansen-Olesen I, 2019. PACAP-38 and PACAP(6–38) degranulate rat meningeal mast cells via the orphan MrgB(3)-receptor. Front. Cell. Neurosci. 13, 114. [PubMed: 30983973]

Piggins HD, Stamp JA, Burns J, Rusak B, Semba K, 1996. Distribution of pituitary adenylate cyclase activating polypeptide (PACAP) immunoreactivity in the hypothalamus and extended amygdala of the rat. J. Comp. Neurol. 376, 278–294. [PubMed: 8951643]

Prescott CA, Kendler KS, 1999. Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. Am. J. Psychiatr. 156, 34–40. [PubMed: 9892295]

Rajbhandari AK, Octeau CJ, Gonzalez S, Pennington ZT, Mohamed F, Trott J, Chavez J, Ngyuen E, Keces N, Hong WZ, Neve RL, Waschek J, Khakh BS, Fanselow MS, 2021. A basomedial amygdala to intercalated cells microcircuit expressing PACAP and its receptor PAC1 regulates contextual fear. J. Neurosci. 41, 3446–3461. [PubMed: 33637560]

Ramikie TS, Ressler KJ, 2016. Stress-related disorders, pituitary adenylate cyclase-activating peptide (PACAP)ergic system, and sex differences. Dialogues Clin. Neurosci. 18, 403–413. [PubMed: 28179812]

Resch JM, Boisvert JP, Hourigan AE, Mueller CR, Yi SS, Choi S, 2011. Stimulation of the hypothalamic ventromedial nuclei by pituitary adenylate cyclase-activating polypeptide induces hypophagia and thermogenesis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R1625–R1634. [PubMed: 21957159]

Ressler KJ, Mercer KB, Bradley B, Jovanovic T, Mahan A, Kerley K, Norrholm SD, Kilaru V, Smith AK, Myers AJ, Ramirez M, Engel A, Hammack SE, Toufexis D, Braas KM, Binder EB, May V, 2011. Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. Nature 470, 492–497. [PubMed: 21350482]

Rosas M, Zaru A, Sabariego M, Giugliano V, Carboni E, Colombo G, Acquas E, 2014. Differential sensitivity of ethanol-elicited ERK phosphorylation in nucleus accumbens of Sardinian alcohol-preferring and -non preferring rats. Alcohol 48, 471–476. [PubMed: 24877898]

Ross RA, Hoeppner SS, Hellberg SN, O'Day EB, Rosencrans PL, Ressler KJ, May V, Simon NM, 2020. Circulating PACAP peptide and PAC1R genotype as possible transdiagnostic biomarkers for anxiety disorders in women: a preliminary study. Neuropsychopharmacology 45, 1125–1133. [PubMed: 31910434]

Saba L, Porcella A, Congeddu E, Colombo G, Peis M, Pistis M, Gessa GL, Pani L, 2001. The R100Q mutation of the GABA(A) alpha(6) receptor subunit may contribute to voluntary aversion to ethanol in the sNP rat line. Brain Res. Mol. Brain Res. 87, 263–270. [PubMed: 11245930]

Sabino V, Cottone P, Blasio A, Iyer MR, Steardo L, Rice KC, Conti B, Koob GF, Zorrilla EP, 2011. Activation of sigma-receptors induces binge-like drinking in Sardinian alcohol-preferring rats. Neuropsychopharmacology 36, 1207–1218. [PubMed: 21346735]

Sabino V, Cottone P, Koob GF, Steardo L, Lee MJ, Rice KC, Zorrilla EP, 2006. Dissociation between opioid and CRF1 antagonist sensitive drinking in Sardinian alcohol-preferring rats. Psychopharmacology (Berl) 189, 175–186. [PubMed: 17047935]

Sabino V, Cottone P, Zhao Y, Iyer MR, Steardo L Jr., Steardo L, Rice KC, Conti B, Koob GF, Zorrilla EP, 2009. The sigma-receptor antagonist BD-1063 decreases ethanol intake and reinforcement in animal models of excessive drinking. Neuropsychopharmacology 34, 1482–1493. [PubMed: 18946467]

Schuckit MA, 1994. Low level of response to alcohol as a predictor of future alcoholism. Am. J. Psychiatr. 151, 184–189. [PubMed: 8296886]

Schuckit MA, 1998. Biological, psychological and environmental predictors of the alcoholism risk: a longitudinal study. J. Stud. Alcohol 59, 485–494. [PubMed: 9718100]

Seiglie MP, Huang L, Cottone P, Sabino V, 2019. Role of the PACAP system of the extended amygdala in the acoustic startle response in rats. Neuropharmacology 160, 107761. [PubMed: 31493466]

Seiglie MP, Smith KL, Blasio A, Cottone P, Sabino V, 2015. Pituitary adenylate cyclase-activating polypeptide induces a depressive-like phenotype in rats. Psychopharmacology (Berl) 232, 3821–3831. [PubMed: 26264905]

Sherwood NM, Krueckl SL, McRory JE, 2000. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocr. Rev. 21, 619–670. [PubMed: 11133067]

Sigvardsson S, Bohman M, Cloninger CR, 1996. Replication of the stockholm adoption study of alcoholism. Confirmatory cross-fostering analysis. Arch. Gen. Psychiatr. 53, 681–687. [PubMed: 8694681]

Sommer W, Hyytia P, Kiianmaa K, 2006. The alcohol-preferring AA and alcohol-avoiding ANA rats: neurobiology of the regulation of alcohol drinking. Addiction Biol. 11, 289–309.

Stojakovic A, Ahmad SM, Malhotra S, Afzal Z, Ahmed M, Lutfy K, 2020. The role of pituitary adenylyl cyclase-activating polypeptide in the motivational effects of addictive drugs. Neuropharmacology 171, 108109. [PubMed: 32325064]

Szabó G, Mácsai M, Schek E, Telegdy G, 1998. The effect of vasoactive intestinal polypeptide and pituitary adenylate cyclase activating polypeptide on tolerance to morphine and alcohol in mice. Ann. N. Y. Acad. Sci. 865, 566–569. [PubMed: 9928071]

Tanaka K, Hashimoto H, Shintani N, Yamamoto A, Baba A, 2004. Reduced hypothermic and hypnotic responses to ethanol in PACAP-deficient mice. Regul. Pept. 123, 95–98. [PubMed: 15518898]

Tanaka K, Kunishige-Yamamoto A, Hashimoto H, Shintani N, Hayata A, Baba A, 2010. Increased ethanol preference and serotonin 1A receptor-dependent attenuation of ethanol-induced hypothermia in PACAP-deficient mice. Biochem. Biophys. Res. Commun. 391, 773–777. [PubMed: 19944672]

Taylor CR, Levenson RM, 2006. Quantification of immunohistochemistry–issues concerning methods, utility and semiquantitative assessment II. Histopathology 49, 411–424. [PubMed: 16978205]

Timme NM, Linsenbardt D, Timm M, Galbari T, Cornwell E, Lapish C, 2020. Alcohol-preferring P rats exhibit aversion-resistant drinking of alcohol adulterated with quinine. Alcohol 83, 47–56. [PubMed: 31542609]

Varodayan FP, Minnig MA, Steinman MQ, Oleata CS, Riley MW, Sabino V, Roberto M, 2020. PACAP regulation of central amygdala GABAergic synapses is altered by restraint stress. Neuropharmacology 168, 107752. [PubMed: 31476352]

Walker BM, Koob GF, 2007. The gamma-aminobutyric acid-B receptor agonist baclofen attenuates responding for ethanol in ethanol-dependent rats. Alcohol Clin. 31, 11–18

Willuhn I, Burgeno LM, Groblewski PA, Phillips PE, 2014. Excessive cocaine use results from decreased phasic dopamine signaling in the striatum. Nat. Neurosci. 17, 704–709. [PubMed: 24705184]

Zhang L, Hernandez VS, Gerfen CR, Jiang SZ, Zavala L, Barrio RA, Eiden LE, 2021. Behavioral role of PACAP signaling reflects its selective distribution in Exp. Res. glutamatergic and GABAergic neuronal subpopulations. Elife 10.

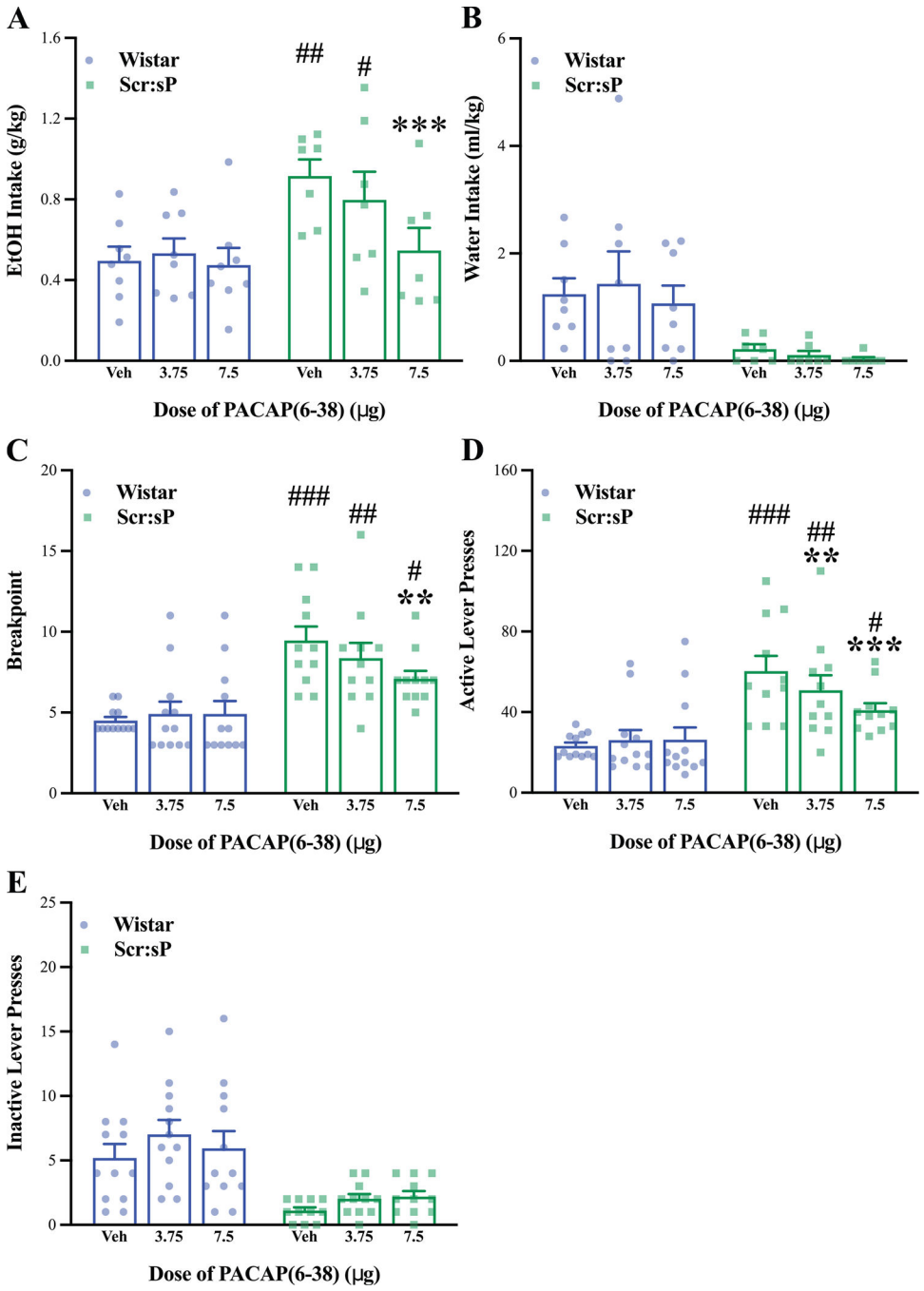

Fig. 1.

A–B: Effect of i.c.v. administration of the PAC1R antagonist PACAP(6–38) (n = 7–8/group) on (A) ethanol intake and (B) water intake in a fixed ratio-1 schedule of reinforcement, in Scr:sP rats and Wistar rats. PACAP(6–38) selectively decreased ethanol intake without affecting water intake, only in Scr:sP rats. Data represent Mean ± SEM. ***p ≤ 0.001 vs. Scr:sP vehicle-treated group; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ vs. Wistar vehicle-treated group. C–E: Effect of i.c.v. administration of PACAP(6–38) (n = 11–14/group) on (C) breakpoint, (D) active lever presses, and (E) inactive lever presses, in a progressive schedule of

reinforcement in Scr:sP rats and Wistar rats. PACAP(6–38) significantly decreased both breakpoint and active lever responses selectively in Scr:sP rats. Data represent Mean ± SEM. **p ≤ 0.01, ***p ≤ 0.001 vs. Scr:sP vehicle-treated group; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ (Duncan's test) vs. Wistar vehicle-treated group.

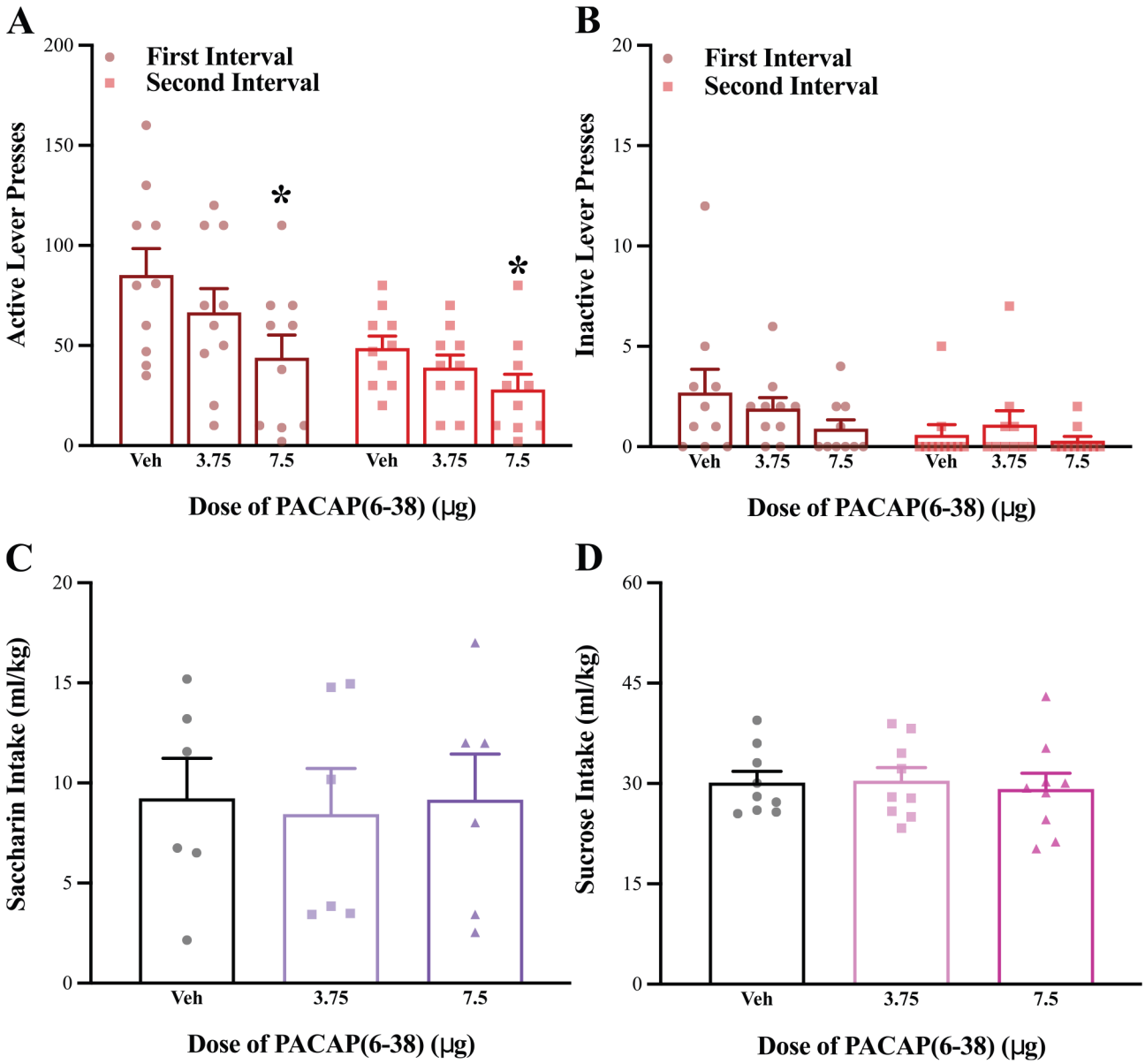

Fig. 2.

Effect of i.c.v. administration of the PAC1R antagonist PACAP(6–38) ($n = 10$) on the number of (A) active lever presses and (B) inactive lever presses during the first (pre-ingestive) interval, and during the second (post-ingestive) interval in a second-order schedule of reinforcement to assess alcohol-seeking behavior in Scr: sP rats. PACAP(6–38) significantly decreased active lever presses responses without affecting inactive. Effect of i.c.v. administration of PACAP(6–38) on (C) saccharin intake ($n = 6$) and (D) sucrose intake ($n = 9$) in a fixed ratio-1 schedule of reinforcement in Scr:sP rats. PACAP(6–38) did not affect saccharin or sucrose intake. Data represent Mean ± SEM. *p ≤ 0.05 (Duncan's test) vs. vehicle-treated group.

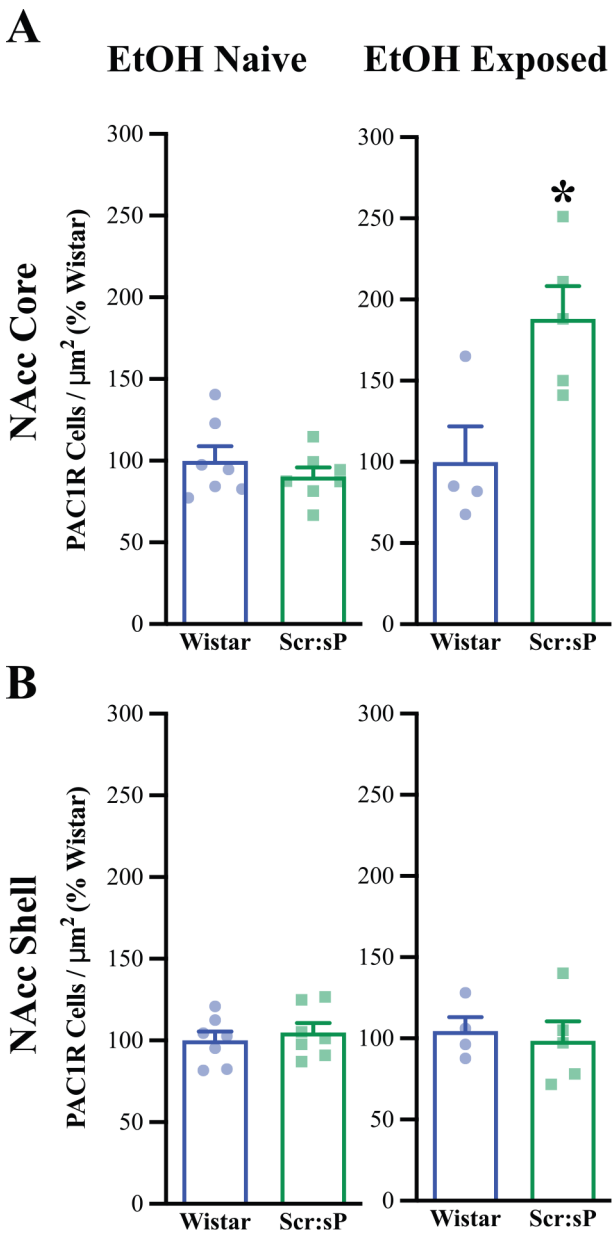

Fig. 3.

PAC1R expression in the NAcc Core (A) and Shell (B) of Scr:sP rats vs. Wistar rats in ethanol-naïve (n = 7/group) and ethanol-exposed (n = 4–5/group) animals. PAC1R expression in the NAcc Core and Shell was not significantly different in ethanol-naïve Wistar and Scr:sP rats. A significant increase in PAC1R expression was found in the NAcc Core in ethanol-exposed Scr:sP rats. No significant difference was found in NAcc Shell in ethanol-exposed rats. Data represent Mean ± SEM intake; *p ≤ 0.05 (t-test) vs. Wistar group.

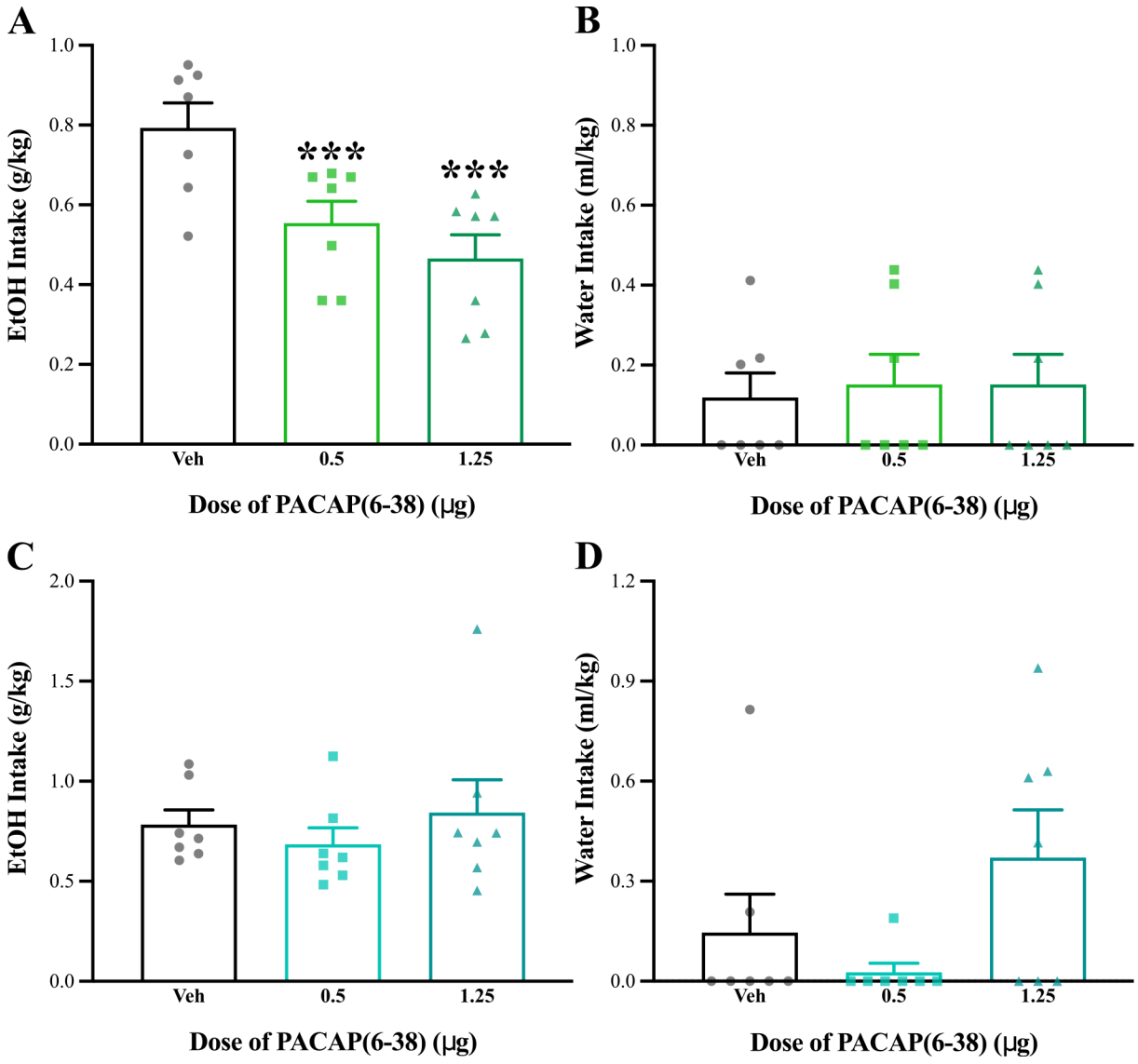

Fig. 4.

Effect of the PAC1R antagonist PACAP(6–38) microinfusion in the NAcc Core ($n = 7$) on (A) ethanol intake and (B) water intake, and in the NAcc Shell ($n = 7$/group) on (C) ethanol intake and (D) water intake, in a fixed ratio-1 schedule of reinforcement in Scr:sP rats. PACAP(6–38) significantly reduced ethanol, but not water intake, when microinfused in the NAcc Core. Data represent Mean ± SEM. ***$p \leq 0.001$ (Dunnett's test) vs. vehicle-treated group. See Sup. Fig. 1 for illustrations of coronal rat brain slices with cannula placement sites.

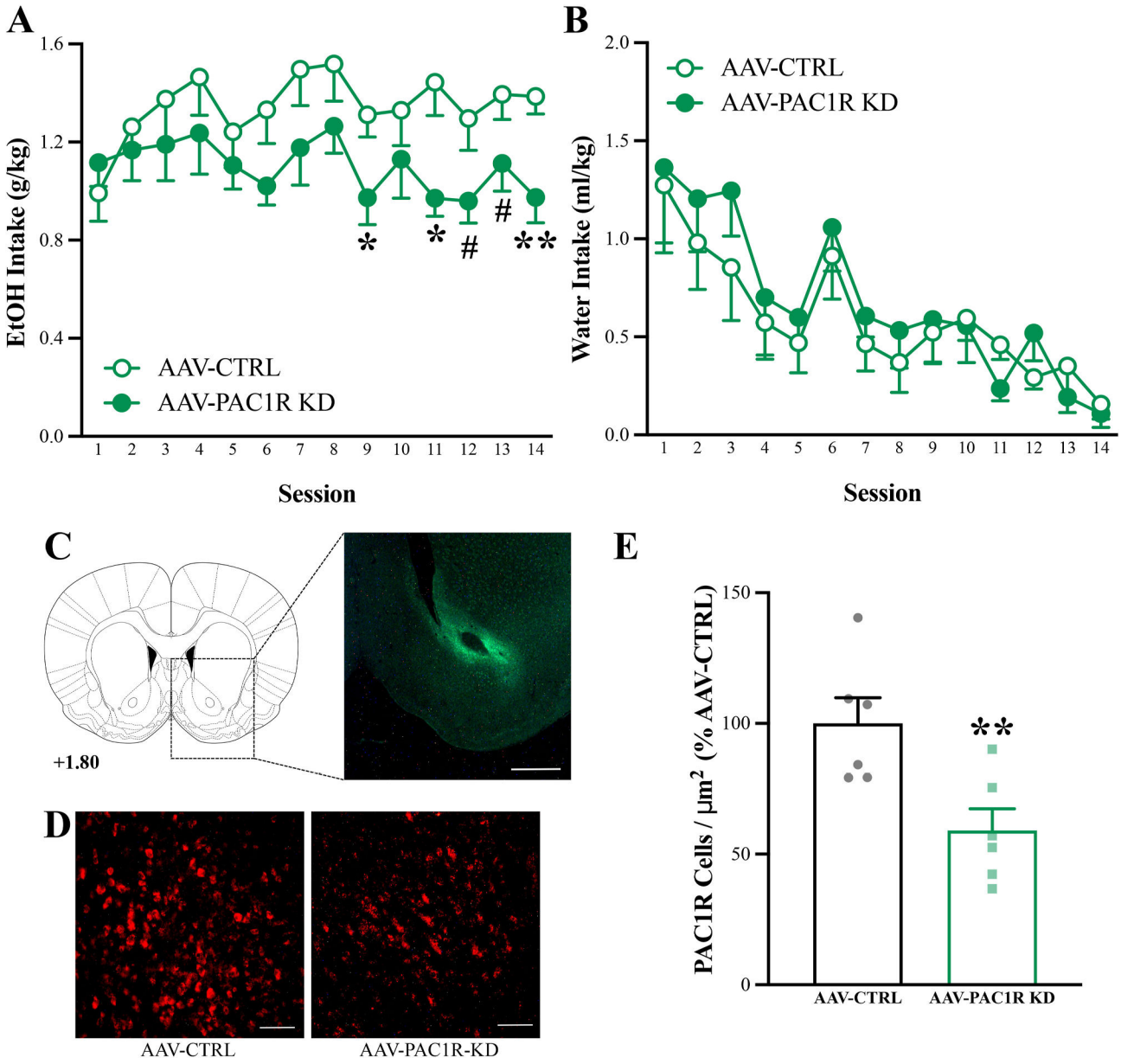

Fig. 5.

Effect of AAV-mediated short-hairpin RNA PAC1R knockdown in the NAcc Core in Scr:sP rats on (A) ethanol intake and (B) water intake, as compared to Scr: sP rats injected with a control virus (n = 8–10/group). (C) Representative viral spread. Confirmation of reduced PAC1R immunoreactivity in shRNA knockdown rats (D, E), compared to controls (n = 6/group). Data represent mean ± SEM. #$p \leq 0.1$, *$p \leq 0.05$, **p ≤ 0.01 (t-test) vs. AAV-CTRL control group.