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Pituitary adenylate cyclase-activating polypeptide regulates excessive alcohol consumption

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PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE
REGULATES EXCESSIVE ALCOHOL CONSUMPTION

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PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE REGULATES EXCESSIVE ALCOHOL CONSUMPTION

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ABSTRACT
Alcoholism results from an interaction between genetic and environmental factors. However, the neurobiological mechanisms mediating the propensity to consume excessive amounts of alcohol are still not well understood. Using genetically selected alcohol-preferring rats, a well-established animal model of alcoholism, we demonstrate that central administration of a peptide antagonist for the pituitary adenylate cyclase-activating polypeptide receptor 1 (PAC1), the cognate receptor for the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), blocks excessive alcohol drinking as well as motivation to drink. On the other hand, the PAC1 antagonist does not significantly affect water intake, saccharin intake, or responding for ethanol in non-selected outbred Wistar rats. In addition, the antagonist significantly reduced responding maintained by alcohol-associated incentive stimuli (alcohol seeking behavior). Using immunohistochemistry, a significant reduction in the number of PAC1 positive cells was observed selectively in the Nucleus Accumbens (NAcc) Core of alcohol-preferring compared to Wistar rats. Proving the functional relevance of these changes, excessive drinking in alcohol-preferring rats was markedly
reduced following microinfusion of the PAC1 antagonist into the Core, but not the Shell, of the NAcc. Finally, using retrograde tracing techniques coupled with immunofluorescence, we show that the dopaminergic neurons of the VTA which project to the NAcc core co-express PACAP. Altogether, our findings demonstrate that the dysregulation of the PACAP/PAC1R system, specifically in the NAcc core, promotes excessive drinking and alcohol-seeking behavior, indicating that blockade of the PACAP/PAC1R system may represent a novel target for alcohol addiction.
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LIST OF ABBREVIATIONS

BNST……………………………………………..Bed nucleus of the stria terminalis
CRF……………………………………………………..Corticotropin-releasing factor
NAcc……………………………………………………Nucleus accumbens
PVN……………………………………………………Paraventricular nucleus
PACAP………………………………Pituitary Adenylate Cyclase Activating Peptide
VTA…………………………………………………..Ventral Tegmental Area
INTRODUCTION

Alcohol Addiction

Alcohol addiction is a debilitating disorder that affects over 140 million people worldwide (1). In 2002, the World Health Organization estimated that alcohol causes 1.8 million deaths and 58.3 million of disability-adjusted life years; the organization has also stated that patterns of heavy drinking exist in most developed countries (1). To put this in perspective, alcohol has a greater toll on world health than tobacco and is on par with unsafe sex, measles, and malaria. According to the Center of Disease Control, between 2006 and 2010 over 87,000 deaths per year, which translates into 1 in 10 deaths in the same time span in the United States, were attributed to alcohol-related issues (67). The economic pitfall of alcohol abuse in 2006 was estimated to be $223.5 billion (67).

Alcoholism is characterized by uncontrolled heavy drinking as well as craving and seeking. Its etiology is complex, as heritable susceptibility factors contribute to 50-60% of the disease risk and interact with environmental factors that produce and maintain the disorder, pointing to specific genotypes that are particularly susceptible to addiction and addictive behavior (4). Genetic factors and neuroadaptations resulting from continuous alcohol consumption can, in fact, act independently to generate the same phenotypes, indicating that the alcohol addiction phenotype can be acquired through slow changes in neural pathways with consistent exposure to alcohol; the molecular correlates of the genetic
propensity to drink can be duplicated environmentally by chronic ethanol exposure.

**Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)**

In this context, we investigated the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP). Originally isolated from the ovine hypothalamus (10), PACAP is encoded by the Adcyap1 gene. PACAP belongs to a superfamily which includes growth hormone-releasing hormone, secretin, and vasoactive intestinal peptides, and it exists in two different fragments, PACAP-38 and PACAP-27 (10, 11). The most abundant population of PACAP-containing neurons, as well as the highest density of PACAP binding sites, is found in the hypothalamus; PACAP-containing neurons are also found in the bed nucleus of the stria terminalis and the amygdala (62). The neurons are found mainly in the paraventricular and supraoptic nuclei. Importantly, Three PACAP receptors have been characterized; two of them, VPAC1 and VPAC2, recognize both VIP and PACAP with equal affinity, while the third, PAC1, is a PACAP-selective receptor (12). Interestingly, the PACAP antagonist PACAP(6-38) blocks PAC1 receptors and does not interact with VPAC1, but does have an affinity for VPAC2. The large amount of immunoreactivity in the hypothalamus for PACAP suggests that PACAP plays a role in many hypothalamically regulated processes. Indeed, the PACAP/PAC1 system has been demonstrated to regulate food intake, energy metabolism, body temperature, and neuronal survival (13-
15). Central administration of PACAP has been shown to reduce feeding in both normal and food-restricted conditions. Furthermore, injections cause rapid elevations in core body temperature and spontaneous locomotor activity (15). Several groups including ours have shown that the PACAP system is a strong mediator of the behavioral response to stress. PACAP has been shown to increase substantially in the anterolateral BNST of rats exposed to chronic variable stress compared to non-stressed rats.

PACAP fibers were also shown to innervate hypothalamic CRF neurons in the praventricular nucleus (PVN) and extrahypothalamic CRF neurons in the BNST (16, 17). PACAP has been linked to the production of CRF as administration of PACAP causes increase in CRF gene expressions (17, 63). The anxiogenic and anti-rewarding effects of PACAP have been shown to be mediated by the recruitment of the central CRF system, especially in the central amygdala (17). However, PACAP also acts independently of the CRF system. PACAP induces an anorectic effect, causing a dramatic reduction in food intake and body weight; this effect is not blocked by CRF receptor antagonist, suggesting that some effects of PACAP do not involve the CRF system (17). Based on these findings, it is clear that PACAP plays a significant role in the stress response system both dependent and independently of CRF.
Alcohol Addiction and the PACAP/PAC1 System

A growing body of evidence links the PACAP/PAC1 system with the actions of drugs of abuse and alcohol. Morphine-induced motor stimulation and conditioned place preference are potentiated by low doses of PACAP, and they are blunted in PACAP-deficient mice compared to their wild-type counterparts, suggesting that endogenous PACAP positively modulates the acute actions of morphine (20). It is also found that PACAP-deficient mice display an increase in basal motor activity, and PAC1 receptor deletion results in a similar increase in basal motor activity; the effect of exogenous PACAP on motor stimulation suggests that this abnormal behavior displayed in PACAP-deficient mice is due to the lack of peptide in the brain’s reward and stress system (20). It is likely that PAC1 receptors play a role in PACAP’s inhibition on motor activity (20). Studies on the intoxicating and ataxic effects of ethanol in Drosophila led to the identification of a mutation (named “Cheapdate”) with higher sensitivity to ethanol-induced ataxia, due to an allele of the gene that encodes PACAP (21).

PACAP signaling likely mediates the response that produces the acute tolerance to ethanol-induced ataxia, diminishing the intoxication actions of ethanol. Thus, high amounts of PACAP may lead to a higher preference for alcohol, as less-impaired motor control during the early stages of drinking seems linked to a greater risk of long-term alcohol addiction (22, 23). Those that experience a lower level of intoxication at lower blood alcohol levels may have a greater likelihood of consuming larger amounts in order to obtain effects similar
to those experienced by others with lower amounts of alcohol consumed, which would then lead to the development of both behavioral and pharmacological tolerance (22). In addition, acute ethanol increases PAC1 receptor mRNA levels via RACK1 (24), which most likely results in an enhancement in PACAP signaling. The PAC1 gene contains an AP-1 site upstream of its promoter region (24), a transcription factor known to regulate gene expression in response to stress, which may explain the effect of ethanol on the PAC1 mRNA levels. PACAP is also able to counteract the toxic effects of ethanol in cerebellum in vivo while also attenuating the expression of pro-apoptotic genes that are increased by alcohol (25). PACAP did not modify plasma alcohol levels and administering the peptide early in the life of the rats was enough to counteract the toxic effects of alcohol and improved cerebellar function (25). Importantly, not only does PACAP reduce the amount of neuronal cell death caused by alcohol, but it also promotes cell differentiation, a key element in functional recovery from damage caused by ethanol intoxication (25). Interestingly, PACAP modulates the hypothermic and hypnotic effects of ethanol; PACAP<sup>−/−</sup> mice were less sensitive in both effects than wild-type mice to intoxicating blood ethanol levels (26). PAC1 receptors show normal sensitivity to ethanol, and since PAC1 receptor-deficient mice showed normal sensitivity to ethanol, the VPAC receptors are likely responsible for the reduced sensitivity to ethanol in the PACAP<sup>−/−</sup> mice. Furthermore, mice deficient in a subunit of protein kinase A showed low sensitivity to intoxicating levels of ethanol in the blood, suggesting that the cyclic
AMP-protein kinase A system, the main pathway of the VPAC receptors, is involved in ethanol response (68).

Remarkably, a single nucleotide polymorphism of ADCYAP1 (Asp54Gly) with potential relevance to alcohol drinking (g/kg/week) was identified in a Finnish population of social drinkers (the recessive model of the GG genotype predisposes individuals to higher levels of intake); individuals of this genotype showed a significant increase in risk of alcohol dependence as compared to the wild-type (27). Those with the recessive genotype required a larger number of drinks needed to obtain an effect of the alcohol during their first five times drinking ever, suggesting those with the genotype have low sensitivity to alcohol (27).

PACAP cell bodies and fibers are found in high quantities in hypothalamic nuclei, as well as in various extra-hypothalamic regions. PACAP-38 mRNA was also found in high amounts in the hypothalamus, suggesting that PACAP is processed and released close to where it is synthesized; strikingly, PACAP is not found in the rat anterior pituitary gland, suggesting it is not locally synthesized within the pituitary but rather is released through neurons from different sites and thus resembles other hypophysiotrophic hormones in effect (28). The nucleus accumbens (NAcc) and the ventral tegmental area (VTA) are among the brain regions expressing the highest levels of PACAP, specifically PACAP-38, immunoreactivity (28), suggesting that the PACAP/PAC1 system might interact
with the function of the mesolimbic dopaminergic system implicated in the 
rewarding effects of alcohol.

The relationship between the PACAP/PAC1 system and alcohol 
reinforcement and addiction remains unclear. We used a line of alcohol-
preferring Sardinian rats, genetically selected for alcohol preference and 
consumption, to unravel the neurobiological factors underlying the predisposition 
to consume excessive quantities of alcohol, an effective approach often used in 
preclinical research (8, 9). These rats exhibit the fundamental signs of 
alcoholism: they ingest alcohol orally, voluntarily consume doses of alcohol that 
result in pharmacologically significant blood alcohol levels, which suggests that 
they do not consume alcohol due to taste, smell, or caloric properties, are willing 
to work for alcohol, e.g. lever-pressing, to obtain alcohol, and voluntarily 
consume alcohol to an extent such that tolerance is able to develop (65). 
However, it is not clear whether or not they develop signs of physical 
dependence after voluntary alcohol consumption and the alcohol is removed. A 
likely explanation for this behavior, which is considerably different from the wild-
type, is the difference in genotypes that causes these rats to have high alcohol 
preference and consumption.

The present studies aimed to determine if the PACAP/PAC1 system 
played a role in alcohol reinforcement by exploring whether or not the PACAP 
antagonist PACAP(6-38) would reduce excessive alcohol intake during operant 
conditioning in alcohol-preferring rats, as well as determining the selectivity of the
effect of PACAP by testing the antagonist PACAP(6-38) with saccharin intake in alcohol-preferring rats. The present studies also aimed to explore the role of the PACAP/PAC1 system in motivation for alcohol in alcohol-preferring rats by determining the efficacy of the antagonist PACAP(6-38) in a progressive ratio test of operant conditioning. Finally, the present studies aimed to study the neurobiology of the PACAP fibers and PAC1 receptors in the brain, especially in the VTA and NAcc, as well as to explore any differences between in the inherent neurobiology of alcohol-preferring rats and outbred Wistar rats.
METHODS

Subjects

Subjects of this study were male Wistar rats, 225-250 grams 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 for 12 generations at Boston University without further selective breeding. Scr:sP rats were generated from intra-line breeding at The Scripps Research Institute from sP rats generously provided after 32 generations of selective breeding from Prof. G. L. Gessa (University of Cagliari, Italy). Rats were chosen for breeding only if their alcohol intake was 4 g/kg or higher and they showed a preference ratio of twice the amount of alcohol intake as compared to water intake. Subjects were housed in an AAALAC-approved vivarium on a 12-h light-dark cycle (lights off at 9:00 a.m.), 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 of 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.
Drugs

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 hydrate (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) and was administered 30 minutes before the experimental sessions. Testing began when performance stabilized (<20% variation across three consecutive sessions). Experiments followed a within-subject design and at least two treatment-free days were allowed between treatment days.

Intracranial surgery and mincroinfusion procedure

Surgeries: The surgical procedures were performed as previously described (1, 2). Rats underwent unilateral (for intracerebroventricular (i.c.v.) experiments) or bilateral (for Nucleus Accumbens (NAcc) core and shell) implantation for 24-gauge stainless steel cannulas (Plastics One, Roanoke, VA) under stereotaxic control (Kopf Instruments, Tujunga, CA), using the following coordinates (from bregma, in mm, DV from skull): i.c.v.: AP -1.0 ML: ± 1.5, DV: -2.3. NAcc core: AP: +1.4, ML: ± 2.4, DV: -5.6; NAcc shell: AP: +1.06, ML: ±0.75, DV: -5.7. NAcc core surgery was performed with a <6 degree angle to avoid the
ventricles. The incisor bar was set at -3.3 mm from the interneural line for all surgeries.

*Microinfusion procedure:* For i.c.v. and site-specific microinfusions, the dummy stylet was removed from the guide cannula and was replaced with a 31-gauge stainless steel injector projecting 2.5mm and 1.5mm, respectively, beyond the tip of the guide cannula, which was connected via a polyethylene 20 tubing to a Hamilton microsyringe driven by a microinfusion pump (KD Scientfics, Holliston, MA). Two days prior to test days, subjects were shammed to simulate injections using 31-gauge stainless steel injectors projecting 2.5mm and 1.5mm, respectively, beyond the tip of the guide cannula, which was connected to an empty polyethylene tubing. Microinjections were performed in a 5 µl (for i.c.v.) and 0.5 µl/side (for NAcc) volume, delivered over one minute; injectors were left in place for an additional minute to minimize backflow. I.c.v. cannula placement was functionally verified at the end of experiments as a positive dipsogenic response (>5ml water intake within 15 min) to an i.c.v. injection of angiotensionII (25 ng/5 µl). For verification of site-specific cannula placement, subjects were microinfused with India ink (0.5 µl/side); coronal sections of a 40 µl were collected using a cryostat and placements were verified under a microscope.

**Apparatus for operant oral ethanol/saccharin self-administration**

The test chambers used for operant oral self-administration (Med Associates, Inc., St. Albans, VT) were located in sound-attenuating, ventilated
cubicles. Two separate syringe pumps (Med Associates, St. Albans, VT) were used: one that dispensed ethanol or saccharin into the right-side stainless steel drinking cup mounted 2 centimeters above the grid floor, and one that dispensed water into the left-side drinking cup. Both cups were located in the middle of one side panel. Two retractable levers were located 3.2 cm to either side of the drinking cups. One lever responded with the syringe of ethanol or saccharin and the other responded with the syringe of water. Fluid delivery and operant responses were controlled by microcomputers.

**Self-administration procedure: Fixed Ratio-1 schedule of reinforcement**

Rats were first allowed continuous (24 hr/day) two-bottle choice access to ethanol (10% w/v) and water in their home cages for 1 week, and then allowed limited daily access (2 hr/day) for 3-4 days. Rats were then allowed 1-3 overnight two-choice operant access to ethanol and water with chow available *ad libitum*; sessions lasted for 16 hours and the number of sessions for each subject depended on the amount of lever presses each subject performed in each overnight trial.

For saccharin, rats were trained to self-administer a saccharin solution (0.02% w/v). Rats were then allowed 1-3 overnight two-choice operant access to saccharin and water with chow available *ad libitum*; sessions lasted for 16 hours and the number of sessions for each subject depended on the amount of lever presses each subject performed in each overnight trial. Each response of the
right-side lever resulted in the delivery of 0.1 ml of fluid (ethanol or saccharin) and sessions were 30 minutes in duration. Lever presses had no scheduled consequences if pressed repeatedly within 2.01 seconds after activation of the pumps to avoid double responses. During all sessions, rats were also allowed to press for water on the opposite lever. Each response of the left-side lever resulted in delivery of 0.1 ml of water.

**Self-administration procedure: Progressive Ratio schedule of reinforcement**

Scr:sP and Wistar rats 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 0.1 ml of ethanol (10% w/v) increased with successive deliveries based on the following exponential progression: response ratio = 4 × (e# of reinforcer×0.1) – 3.8, rounded to the nearest integer as in (3). Start of the session was defined upon completion of the first ratio, with the latency to complete the first ratio recorded as a dependent measure, and set to a maximum of 2 hours. To avoid unintended session starts, the first reinforcement required three responses. Thus, the PR schedule was 3, 1, 2, 2, 3, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 16, 18, 20, 23, etc. responses; sessions ended when subjects had not completed a ratio for 14 minutes, as we and others previously reported (3-5), with the last completed ratio
defined as the breakpoint, the point at which the subjects ceases to work for ethanol reward. Responses on the inactive lever were also recorded.

**Alcohol-seeking behavior: Second-order schedule of reinforcement**

Active and inactive levers were counterbalanced throughout the study between left and right sides. Subjects were 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. Responses on the inactive lever had no programmed consequences, but were recorded as an index of motor activity. Lever presses on the active lever resulted in ethanol solution delivery concomitantly with illumination of a conditioned stimulus (CS) light above the active lever for 20 seconds (time out, TO) followed by lever retraction. The session ended when subjects reached 30 rewards or after 2 hours, whichever occurred first. Subjects were then introduced to the next step of the training, a fixed interval (FI) schedule of reinforcement. The FI schedule increased daily from FI1 minute, to FI2, FI4, FI8, and FI10 minutes before stabilizing at FI15 minutes. After the FI schedule, a 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. The session ended when subjects received 30 rewards or after 2 hours. Finally, subjects were moved to a second-order schedule of reinforcement. The second order schedule was comprised of two 15-minute fixed intervals. During the FI15min, every 10th active lever press resulted in a brief CS
presentation for 1 second above the active lever (FI15(FR10:S)); after the FI15 min, ten active lever presses resulted in a delivery of 0.12 ml of ethanol solution and CS presentation for 20 seconds. The session ended when subjects completed two intervals or after 40 minutes, whichever occurred first.

**Immunohistochemistry**

Fixation: rats were anesthetized with isoflurane and transcardially perfused with phosphate buffered saline (PBS) first and then in 4% paraformaldehyde (PFA). Brains were collected, placed in PFA, and stored in a 30% sucrose PBS solution at 4°C until saturation. Brains were then cut into 30 μm coronal sections using a cryostat and subsequently stored in a cryoprotectant at -20°C until processed for immunohistochemistry.

PAC1 immunohistochemistry: every 12\textsuperscript{th} section (240 μm apart; bregma +2.28 to +0.84 mm) of the NAcc Core, Shell, and Dorsal Striatum (DS) regions were collected in a systematic manner and processed for immunohistochemistry. Sections were subject to an antigen retrieval step (6M Urea solution, pH=9.5 in 0.1M Tris HCl at 95°C for 10 min). After rinsing, incubation in 1% hydrogen peroxide TBS solution to quench endogenous peroxidases, additional rinsing and a blocking step (5% normal goat serum, 3% Triton X100, and 5% bovine serum albumin), sections were incubated in an anti-PAC1R primary antibody in blocking solution for 48 h at 4°C (1:250, Santa Cruz, Biotechnology, Dallas, TX). Sections were then rinsed and incubated in an anti-PAC1R primary antibody in in a
biotinylated anti-rabbit secondary antibody (1:200, Vector Labs, Burlingame, CA) and immunoreactivity was visualized using a diaminobenzidine substrate kit (Vector Labs, Burlingame, CA) according to the manufacturer’s instructions. Slides were dehydrated using graded alcohol concentrations and then cover slipped.

Fluorescence immunohistochemistry: We used a retrograde tracing technique coupled with immunofluorescence to determine whether the dopaminergic neurons of the VTA which projected to the NAcc core also contained PACAP. Rats were stereotaxically injected with 0.1 µl of a 4% w/v FluoroGold solution (retrograde axonal tracer) into the NAcc core (6, 7). 7 days later rats were anesthetized, perfused as described above, and brains removed for immunofluorescent quantification of FluoroGold/TH/PACAP triple-labeled neurons. FluoroGold-labeled sections which included the VTA (bregma +2.28 to +0.84 mm) were washed in TBS between each blocking and incubation step. Sections were first placed in a blocking solution(3% normal goat serum and 0.25% bovine serum albumin). Subsequently, sections were incubated for 24 hr with rabbit anti-PACAP (1:500, Bachem, Torrance, CA) and mouse anti-TH (1:500 EMD Millipore, Billerica, MA) in blocking solution. Then for 2 hr with biotinylated goat anti-rabbit (Vector Labs, Burlingame, CA) with Alexa Fluor 488 goat anti-mouse (Jackson ImmunoResearch, West Grove, PA) in blocking solution; finally sections were placed in streptavidin Cy3 (Jackson ImmunoResearch, West Grove, PA) in blocking solution.
Microscopy

Sections from the PAC1 and the fluorescent immunohistochemical experiments were analyzed using an Olympus (Center Valley, PA) BX-51 microscope equipped with a Rotiga 2000R live video camera (Ludl Electronics, Hawthorne, NY), and a EXFO X-cite Series 120 fluorescent illuminator (EXFO, Vanier, Quebec, Canada).

Quantification of PAC1 positive cells were performed in accordance with an unbiased stereology approach. Serial sections were created virtually using the number of sections per slide, the thickness in micrometers of each section, and the actual thickness of each section after preparation and dehydration in order to create a virtual 3-D representation of the regions outlined. Each region was outlined virtually and individually on the digitized image of each section using the optical fractionator workflow module of Stereo Investigator software (MicroBrightField, Williston, VT). One hemisphere was randomly chosen for each section; contours were drawn at low magnification using an Olympus PlanApo N 2X objective with numerical aperture 0.08 and counted using Olympus UPlanFL N 40X objective with numerical aperture 0.75. Each group of brains was counted by the same person to avoid differences in perception of cell bodies as well as to minimize discrepancies between cell counts in the brains.
RESULTS

I.c.v. PACAP (6-38) blocked 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 control outbred Wistar rats under an FR1 schedule of reinforcement (panel A vs. panel C; Genotype: F(1, 13)=4.95, p<0.05). I.c.v. administration of the PAC1R antagonist PACAP (6-38) selectively and dose-dependently blocked excessive ethanol self-administration in Scr:sP rats (Fig. 1A), but not in outbred Wistar rats (Fig. 1C), as reflected by a significant interaction of Genotype x Dose (Genotype*Dose: F(2, 26)=3.96, p<0.05; Dose: F(2, 26)=5.51, p=0.01); 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, indeed, 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 and 1D, responding for water was unaffected by PACAP (6-38) in any of the two genotypes (Genotype*Dose: F(2, 26)=1.56, n.s.; Dose: F(2, 26)=0.54, n.s.).
Fig. 1 Effect of i.c.v. administration of PACAP(6-38) (n=7-8/group) on (A-C) ethanol intake and (B, D) water intake in a fixed ratio-1 schedule of reinforcement in Scr:sP rats (A, B) and Wistar rats (C, D). PACAP(6-38) selectively decreased ethanol intake without affecting water intake, selectively in Scr:sP rats. Data represent Mean±SEM. **p≤0.01 vs. Scr:sP vehicle-treated group; *p≤0.05 vs. Wistar vehicle-treated group.
I.c.v. PACAP (6-38) decreased the motivation to drink ethanol in alcohol-preferring, but not in outbred rats

As shown in Fig. 2, Scr:sP rats showed higher breakpoint for ethanol compared to outbred Wistar rats under a progressive ratio schedule of reinforcement (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 (Fig. 2A), but not in outbred Wistar rats (Fig. 2C) (Genotype*Dose: F(2, 42)=3.91, p<0.05; Dose: F(2, 42)=1.97, n.s.). Post hoc analysis showed that the highest dose of PACAP (6-38) significantly reduced the breakpoint in Scr:sP rats (25% of reduction vs. vehicle condition). PACAP (6-38) also reduced the total responses for ethanol selectively in Scr:sP rats (data not shown; Genotype: F(1, 21) = 14.9, p<0.001; Genotype*Dose: F(2, 42)=4.49, p<0.05; Dose: F(2, 42)=2.34, n.s.). The antagonist treatment had no effect on the number of inactive lever presses (Fig. 2B and 2D) (Genotype*Dose: F(2, 42)=0.42, n.s.; Dose: F(2, 40)=1.30, n.s.).
**Fig. 2** Effect of i.c.v. administration of PACAP(6-38) (n=11-14/group) on (A-C) breakpoint and (B-D) inactive lever presses in a Progressive Ratio schedule of reinforcement in Scr:sP rats (A, B) and Wistar rats (B, D). Data represents Mean±SEM. *p≤0.05, **p≤0.01 vs. Scr:sP vehicle-treated group; *p≤0.05, **p≤0.01, ***p≤0.001 vs. Wistar vehicle-treated group.
I.c.v. PACAP (6-38) decreased alcohol-seeking behavior in alcohol-preferring rats

As shown in Fig. 3A, PACAP (6-38) decreased alcohol-seeking behavior in Scr:sP rats (F(2, 18)=3.67, p<0.05; Fig. 3A) under a second-order schedule of reinforcement, in which an alcohol-associated conditioned stimulus maintains alcohol-seeking behavior. The highest dose of PACAP (6-38) significantly decreased the number of lever presses during the first interval (43% of reduction). The treatment had no effect on inactive lever presses (F(2, 18)=0.54, n.s.; Fig. 3B).

I.c.v. PACAP (6-38) had no effect on saccharin intake in alcohol-preferring rats

As shown in Fig. 3C, i.c.v. administration of the PAC1R antagonist PACAP (6-38) did not affect lever pressing for a sweet, saccharin solution in Scr:sP rats in an FR1 schedule of reinforcement (F(2, 10)=0.13, n.s.). As desired, this saccharin concentration (0.02% w/v) maintained levels of responding under vehicle conditions that matched those obtained with ethanol (average of 37.3±4.3 and 39.3±7.9 lever presses for ethanol and saccharin, respectively). Once again, PACAP(6-38) had no effect on concurrent water intake (F(2, 10)=0.71, n.s.)(Fig. 3D).
Fig. 3 Effect of i.c.v. administration of PACAP(6-38) (n=10) on (A) the number of active lever presses and (B) inactive lever presses during the first 15-min interval in a second-order schedule of reinforcement in Scr:sP rats. Effect of i.c.v. administration of PACAP(6-38) (n=6) on (C) saccharin intake and (D) water intake in Scr:sP rats. PACAP(6-38) significantly decreased breakpoint selectively in Scr:sP rats, significantly decreased active, but not inactive, lever press responses in the second-order schedule and did not affect saccharin intake. Data represent Mean±SEM. *p≤0.05, **p≤0.01 vs. Scr:sP vehicle-treated group; *p≤0.05, **p≤0.01, *** p≤0.001, vs. Wistar vehicle-treated group.
PAC1 receptor is decreased in NAcc core of alcohol-preferring rats

As shown in Fig. 4 a significant decrease in PAC1R immunoreactivity (PAC1 positive cells) was observed in the NAcc core of Scr:sP rats, compared to Wistar rats (t(14)=3.71, p<0.002). No significant differences were observed in the NAcc shell (t(14)=1.75, n.s) or the Dorsal Striatum (t(14)=0.87, n.s.), suggesting regional specificity.
Fig. 4. Bar graph shows the number of PAC1 receptor + cells in Scr:sP and Wistar rats in NAcc core (right panel), NAcc shell (center panel) and DS (left panel) (A). Representative micrographs of PAC1 receptor immunoreactivity in NAcc core, NAcc shell, and DS (B). A significant decrease in PAC1 receptor + cells was found in Scr:sP in NAcc core. Data represent Mean±SEM. **p≤0.01 vs. Wistar group.
Dopaminergic neurons of the VTA projecting to the NAcc core co-express PACAP

Using immunofluorescence analysis of the Ventral Tegmental Area (VTA) we labeled (green, Fig. 5A) tyrosine hydroxylase (TH)-positive neurons, PACAP-positive neurons (red, Fig. 5B), as well as neurons containing Fluorogold (blue, Fig. 5C), a retrograde axonal tracer which had been microinfused into the NAcc core 7 days before brain collection. We observed that all TH-positive, i.e. dopaminergic, neurons co-localized with PACAP, and all neurons projecting to the NAcc core express both TH and PACAP, as shown by triple-labeling (Fig. 5E-5G; S1E-S1G).
Fig. 5. Immunofluorescence (10x) of (A) TH-positive neurons, (B) PACAP-positive neurons, and (C) FluoroGold-labeled neurons in the VTA of rats injected in the NAcc core with the retrograde axonal tracer, FluoroGold. (D) TH-positive and FluoroGold-positive neurons, (E) TH-positive and PACAP-positive neurons, (F) PACAP-positive and FluoroGold-positive neurons, (G) triple-labeled neurons.
Fig. 6. Effect of PACAP(6-38) microinfusion in the NAcc core (n=7) on (A) ethanol intake and (B) water intake, and in the NAcc shell on (C) ethanol intake and (D) water intake in Fixed Ratio-1 in Scr:sP rats (n=7/group). PACAP(6-38) significantly reduced ethanol (but not water) intake when microinfused into the NAcc core, but not into the shell. Data represent Mean±SEM. **p≤0.01 vs. vehicle-treated group. (E) Illustrations of coronal rat brain slices; dots represent the injection sites in the NAcc core (left column) and shell (right column) included in the data analysis.
**PACAP(6-38) microinfusion in the NAcc core prevented excessive alcohol drinking in alcohol-preferring rats**

Microinfusions of the PAC1R antagonist PACAP(6-38) into the NAcc core significantly decreased excessive ethanol intake under an FR1 schedule of reinforcement in Scr:sP rats (Fig. 6A, F(2, 12)=25.53, p<0.001). Post hoc analysis revealed that PACAP(6-38) significantly reduced ethanol intake (41% reduction at the highest dose). In contrast, no effects were observed when PACAP(6-38) was microinfused into the NAcc shell, as shown in Fig. 6C (F(2, 12)=1.37, n.s.), supporting the immunohistochemistry findings that the NAcc core PAC1Rs are those involved in the control of excessive alcohol drinking. Water intake was not affected by PACAP(6-38) microinfusion in either subregions of the NAcc (core: $X^2(2, N=7)=0.50$, n.s.; shell: $X^2(2, N=7)=1.68$, n.s., Fig. 6B and 6D). Fig. 6, panel E shows the location of the injection sites in the NAcc core (left column) and the NAcc shell (right column) included in the data analysis.
DISCUSSION

This series of studies demonstrates that i.c.v. administration of the PAC1R antagonist PACAP(6-38) dose-dependently and selectively decreased oral ethanol self-administration in alcohol-preferring Scr:sP rats that drink excessive amounts of alcohol. The PAC1 antagonist also reduced their motivation to work to obtain ethanol, as seen by the decreased breaking point in the progressive ratio test, as well as alcohol-seeking behavior. In contrast, PACAP(6-38) did not reduce ethanol self-administration in control outbred Wistar rats, nor did it affect responding for water or for a saccharin solution, suggesting a specificity in the treatment for the alcohol-preferring Sardinian rats, the model of alcoholism used in these studies, and for alcohol intake. Ethanol naïve Scr:sP rats also showed decreased PAC1R expression in the NAcc core, compared to ethanol-naïve control Wistar rats, which suggests that there are significant underlying neurobiological factors that affect alcohol preference and consumption, as well as vulnerability to alcohol addiction. Finally, blockade of PAC1R in the NAcc core, but not the NAcc shell, was sufficient to prevent excessive ethanol intake in Scr:sP rats, also suggesting a specificity of location in the brain for the treatment. The results collectively support an endogenous role for the NAcc core PACAP/PAC1R system in controlling excessive ethanol intake and alcohol-seeking.
Central infusion of PACAP(6-38) not only blocked excessive drinking in alcohol-preferring rats, bringing levels back to those of control outbred rats, but it also reduced the breakpoint under a PR schedule of reinforcement, a measure of the motivation to obtain a reinforcer in which the influence of local response rates on performance are negligible.

Importantly, PAC1R blockade reduced alcohol-seeking behavior, suggesting an effect on incentive motivational mechanisms controlling ethanol-seeking and intake. To assess this, we used a second-order schedule of reinforcement; this task, used with psychostimulants and opiates (29, 30), is characterized by the maintenance of responding not only by the self-administered drug, but also by the contingent presentation of drug-paired stimuli that serve as conditioned reinforcers of instrumental behavior (31). For example, the operant conditioning would include a tone or light that accompanies the presentation of alcohol via lever press. PACAP(6-38) dose-dependently reduced the number of active lever presses during the first “drug-free” interval of the session, suggesting an effect of PACAP(6-38) on either the incentive motivational mechanisms governing alcohol-seeking and intake or on the impact of alcohol-associated conditioned reinforcer (environmental cues) to maintain responding (30, 31). Importantly, this would provide effective treatment in addiction as many addictions, including alcohol, include cue-induced or environmentally induced binge drinking.
It is interesting to note that the antagonist did not alter self-administration of an equally reinforcing sweet solution (i.e. producing the same number of lever presses as the subject produced for 10% alcohol solution under vehicle conditions), suggesting not only that PACAP(6-38) does not produce malaise-like or other unspecific behaviors, but also that the effects of PACAP(6-38) are selective for alcohol. The specificity of drug treatment effects suggests that the PACAP receptor system plays a functional role when the brain reward system is activated specifically by alcohol but plays only a limited role in the regulation of processes activated by sweet taste; the effect of the treatment must therefore not have an effect through taste or smell or caloric value. A simple reduction in caloric intake by PACAP(6-38) can be excluded based on the extensive literature showing that PACAP itself (and not PACAP receptor antagonists) potently reduces food intake.

The observed selective effect of the PAC1R antagonist in alcohol-preferring Scr:sP rats led us to hypothesize that neuroadaptations in PAC1R could be responsible for the excessive drinking phenotype. Using immunohistochemistry we found that PAC1R protein levels were significantly lower (-38%) in the NAcc core of Scr:sP rats, compared to outbred Wistar rats. Similar changes were not observed in NAcc shell or Dorsal Striatum, suggesting regional specificity. The present experiments have, therefore, identified a pre-existing difference in the PACAP/PAC1R system in the NAcc core of Scr:sP rats as compared to their own outbred stock, which is associated with their genetic
propensity to consume alcohol as well as propagate alcohol-seeking behavior. Other cases of increased sensitivity to antagonists associated with decreased density or coupling of the respective receptor have been reported (34, 35). If PACAP is considered a mediator of brain reward function, a parallel can be drawn between a hypo-PACAPergic state and the hypo-dopaminergic state widely reported for alcoholic patients as well as alcohol-preferring rats and linked to the vulnerability to consume excessive amounts of alcohol (36, 37). It can be debated, then, that the low levels of PACAP would be due to a shift into the negative reinforcement phase and therefore a transition into addiction. With less PACAP or PAC1Rs, the baseline for activation of the reward circuit would be higher due to a lower effect of the PACAP stress response, thus not feeling the negative reinforcement due to PACAP. Low levels of PAC1Rs in Scr:sP rats may predispose subjects to drink excessively as a means of compensating for the decrease in activation of downstream reward circuits and therefore, for a deficit in PACAP-regulated reward circuit functions since more alcohol would be needed to activate the reward circuit to the degree of a wild-type subject. Finally, albeit basally reduced, the system may be hyper-responsive to alcohol, like other systems (38). In this study we measured PAC1R levels in alcohol-naïve rats, rather than in rats self-administering ethanol, because ethanol can be a confounding factor while attempting to identify differences between genotypes (32, 33); it would be valuable in future studies to also examine brain changes resulting from alcohol drinking. An alternative hypothesis may involve the re-
sensitization of the PAC1 receptor by the antagonist and therefore an improvement of the PAC1-mediated reward function upon administration of the PAC1 antagonist. This hypothesis is supported by in vitro evidence that chronic administration of PACAP desensitizes PACAP-induced cAMP accumulation, while PACAP(6-38) re-sensitizes it in the chicken retina (39).

In support of the functional significance of the observed dysregulation of PAC1R in the NAcc core of Scr:sP rats and in agreement with our predictions, excessive ethanol responding in Scr:sP rats was suppressed following PACAP(6-38) microinfusion into the NAcc core, but not into the shell. The reduction of ethanol responding following intra-NAcc core administration was observed at doses which were significantly lower (0.5 and 1.25 μg/side) than those which reduced ethanol self-administration following i.c.v. administration (7.5 μg/rat), suggesting that the Scr:sP rats were inherently more sensitive to the treatment due to a decreased level of PAC1Rs in the NAcc core as compared to the reduced ethanol self-administration group.

The NAcc core has been suggested to be a mediator of Pavlovian influences on instrumental action (40, 41). A role for the NAcc core in the acquisition of drug seeking maintained by drug-associated cues has been suggested; furthermore, evidence supports the development of neuroadaptations in the NAcc core following alcohol (and drug) consumption over extended periods of time, suggesting neuroplasticity (42-44). Human imaging studies with
detoxified alcoholics have also shown activation of the NAcc core during exposure to alcohol-associated cues (45).

Importantly, the immunofluorescence data demonstrate in vivo that dopaminergic neurons of the VTA also express PACAP, and that VTA neurons projecting to the NAcc core are indeed DA/PACAP neurons, suggesting a relationship between dopamine and PACAP pathways. In vitro work indicated that PACAP increases transcription of the genes of both tyrosine hydroxylase (TH), a key enzyme in the production of dopamine, and vesicular monoamine transporter (VMAT), a transport protein necessary for transporting dopamine into vesicles for synaptic transmission, via PAC1 (46, 47). Furthermore, PACAP acts as a secretagogue to elicit catecholamine secretion in chromaffin cells and PC12 cells (48 49). And it is involved in dexamethasone-induced dopamine biosynthesis (50). Only a portion of the TH-positive VTA neurons expressed the retrograde tracer FluoroGold and were therefore projections to the NAcc core; this was expected as dopaminergic neurons of the VTA are known to project other areas, including the NAcc shell, prefrontal cortex, and central amygdala (51). In the VTA, co-localizations of TH with other neuropeptides have been demonstrated (e.g. neurotensin and cholecystokinin (52)). Due to the nearly complete overlap between dopaminergic and PACAPergic neurons of the VTA projecting to the NAcc core, PACAP and dopamine may be co-released in this area depending on firing frequencies, as shown in other neuropeptides co-localized with classical neurotransmitters (53, 54).
Based on our data, the likelihood that PACAP is released from VTA mesolimbic neurons in the NAcc following alcohol consumption is high, thereby contributing to the maintenance of high levels of intake via PAC1R activation. Our data lead us to speculate that reduction of excessive ethanol intake after intra-NAcc injection of PACAP(6-38) results from the ability of the antagonist to prevent alcohol-induced potentiation of mesolimbic dopaminergic neurotransmission by PACAP, selectively in alcohol-preferring rats, which would reduce the activity of the PAC1R receptors that activate the reward circuit within the VTA. This hypothesis is in agreement with the observation of higher spontaneous firing rates of dopaminergic VTA neurons in alcohol-preferring rats (55). Therefore, the observed innate dysregulation of the PACAP/PAC1R system in alcohol-preferring Scr:sP rats may be linked to the increased sensitivity of the dopaminergic system to ethanol and, in turn, to the vulnerability to alcohol addiction. With an increased effect of dopamine, the addictive properties of alcohol, i.e. the overstimulation of the brain reward system, would increase alcohol consumption while possibly reducing the stress response system that has been shown to be linked to PACAP and CRF, which would also increase alcohol consumption due to lesser negative consequence.

In conclusion, our behavioral and molecular findings provide evidence that a dysregulation of the PACAP/PAC1R system in the NAcc core is predictive of a vulnerability to drink excessive amounts of alcohol. Importantly, blockade of the PAC1R reveals selectivity in this treatment, as saccharin and water intake were
not affected by the drug. Because of the link between dopaminergic and PACAPergic neurons in the VTA, as shown through our immunohistochemical findings, it is plausible that the PACAP/PAC1R system plays a key role in the development and progression of addiction via its possible role in the brain’s reward system. Blockade of PAC1Rs dramatically reduces consumption, motivation, and seeking of alcohol, as shown by the procedures we have demonstrated in our studies, identifying the PACAP/PAC1R system as a promising therapeutic target for the treatment of alcohol addiction.
## LIST OF JOURNAL ABBREVIATIONS

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CURRICULUM VITAE

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Profile
Accomplished, well-trained, and results-oriented recent Masters Graduate interested in gaining more experience in the scientific research field.

Education

Boston University – Boston, MA
Master of Arts in Medical Science – September 2013 to present
University of California, Los Angeles – Los Angeles, CA
B.S. in Psychobiology – September 2009 to August 2013

WORK Experience

Boston University Laboratory of Addictive Disorders – Boston, MA
Research Assistant – August 2014 to Present

- Responsible for the upkeep and handling of 100+ rat cohorts at any given time
- Conducted operant conditioning sessions using alcohol, sucrose, and saccharin solutions.
- Performed procedures with fixed and progressive ratio and delayed reinforcement learning.
- Experience in preparing solutions, pipetting, and working with blood.
- Collect data for analysis from multiple trials.
- Maintain cleanliness of procedure room as well as laboratory work space.

COPE Clinical Care Extenders – Lynwood, CA
Clinical Intern – September 2009 to June 2013

- Assist nurses and physicians with bathing, feeding, ambulating, and cleaning patients.
- Observe procedures in the Operating Room and received instruction from physicians and anesthesiologists.
- Aid RN’s with taking vital signs of patients and in making patients comfortable.
• Communicate directly with patients and patients’ families to relay information from doctors.

**UCLA Housing and Hospitality – De Neve – Los Angeles, CA**  
*Front Desk Supervisor – January 2011 to April 2013*

• Integral part of customer service team at De Neve dormitories.
• Interacted with and assisted students, faculty, parents, and other UCLA employees.
• Responsible for monetary transactions and trusted with large sums of money.
• Worked together with UCPD, LAFD, and Resident Assistants to ensure student safety.
• Interviewed and trained new employees and upheld high standard for Front Desk employees.
• Maintained clean work environment to make sure Front Desk was presentable.

**Anne McLaren Laboratory for Regenerative Medicine – Cambridge, UK**  
*Front Desk Supervisor – January 2011 to April 2013*

• Worked closely with post-doc and Ph.D. students in stem cell research
• Ran own experiments and used multiple techniques, including Western blot, gel electrophoresis, and pipetting.
• Participated in team discussions and presentations.

**Leadership Experience**

**COPE Clinical Care Extenders – Lynwood, CA**  
*Director of Administration – May 2010 to May 2012*

• Led administrative team in handling intern communications and records.
• Oversaw handling of immunization records and other confidential documents.
• Led reconstruction of Leadership Team system and projects to increase efficiency.
• Assist interns with any problems they face by holding over 8 hours of office hours a week.
• Led intern meetings and discussions, encouraging healthy discussions about policies.

**UCLA Kendo Club – Los Angeles, CA**  
*Captain – September 2011 to May 2013*
• Led team practices and supervised training of beginners.
• Provided guidance for beginning members as well as competitive team members.
• Worked closely with the coach to create lesson plans for each practice.
• Provided input for team events such as travels, recruitment events, and tournament preparation.

**Boy Scouts of America, Troop 911 – Northridge, CA**
*Eagle Scout, Senior Patrol Leader – September 2008 to May 2009*

• Led the largest troop in the West Los Angeles County Council.
• Restructured patrol leadership council for maximum scout participation.
• Increased leadership participation into individual patrol affairs to allow for more communication.
• Guided the development of scouts in skills and ranking.