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Atrial Natriuretic Peptide (ANP): A Novel Mechanism for Reducing Ethanol Consumption and Seeking Behaviors in Female Alcohol Preferring (P) Rats

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Abstract

Atrial Naturietic Peptide (ANP) is a neuropeptide that regulates function of the hypothalamic-pituitary-adrenal (HPA) axis, immune and neuroimmune system, and epigenetic factors. Research has indicated that ANP may mediate alcohol intake, withdrawal, and craving like behaviors. ANP receptors are present in the mesocorticolimbic (MCL) reward pathway of the brain, which includes the nucleus accumbens (Acb) and the ventral tegmental area (VTA). The objectives of the present study were to examine the effects of ANP microinjected into Acb subregions (Shell (Sh), Core (Co), ventral to AcbSh) on operant ethanol (EtOH) self-administration and into posterior VTA (pVTA) on EtOH-seeking behavior of female alcohol-preferring (P) rats. In the first experiment, ANP (0, 10 μg, or 100 μg) was microinjected into subregions of the Acb to determine its effects on EtOH self-administration. In the second experiment, ANP was microinjected into pVTA to determine its effects on Pavlovian Spontaneous Recovery (PSR) of responding, a measure of context-induced EtOH-seeking behavior. Administration of ANP directly into the AcbSh significantly reduced EtOH self-administration compared to vehicle, whereas ANP into the AcbCo or areas directly ventral to the AcbSh did not alter responding for EtOH. Microinjection of ANP into the pVTA significantly reduced responding on the EtOH-associated lever during the PSR test. The data indicate that activation of ANP systems in the (a) AcbSh can inhibit EtOH intake, and (b) in the pVTA can inhibit EtOH-seeking behavior. The results suggest that manipulations of the ANP system could be a potential target for pharmacotherapeutic intervention to treat alcohol use

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Keywords

Atrial Natriuretic Peptide; Alcohol; Alcohol Preferring P rats; Ventral Tegmental Area; Nucleus Accumbens

1. INTRODUCTION

Atrial natriuretic peptide (ANP) is a 28 amino-acid peptide synthesized in the heart atria and brain. ANP is also produced in neurons and glia of the cerebral cortex as well as in neurons and fibers of thalamus, striatum, amygdala, and hippocampus (Hodes and Lichtstein, 2014). ANP has a number of biological actions, including natriuresis, diuresis, vasodilatation, smooth muscle relaxation, as well as inhibition of aldosterone and renin secretion (Potter et al., 2006). In the brain, ANP is considered the apex neuropeptide in the hypothalamic-pituitary-adrenal (HPA) axis. Activation of ANP receptors within the hypothalamus, pituitary, and adrenal cortex inhibits the secretion of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol (Wiedemann et al., 2000). Brain-derived ANP also inhibits the neuropeptide Y (NPY) system and modulates the vasopressin, angiotensin, cholecystokinin (CCK), and other neuropeptide systems (Landgraf, 2005). The corticotropin-releasing factor (CRF) and NPY systems are frequently highlighted in preclinical research for the treatment of alcohol use disorders (AUD), but both have failed to show promise as pharmacotherapeutics for the treatment of AUD in clinical settings (cf., Pomrenze et al., 2017). It may be possible that targeting a neuropeptide that regulates these failed candidates could be successful and may explain the disparity between preclinical and clinical findings.

There is convergent clinical and preclinical genomic data indicating a potential role in the predisposition to consume alcohol. There are three natriuretic peptide-receptors designated natriuretic peptide-A (NPR-A), B (NPR-B), and C (NPR-C), with the effect of ANP being primarily mediated by NPR-A (Garg and Pandey, 2005). Human genome-wide association studies (GWAS) studies have replicated results indicating that a single-nucleotide polymorphism (SNP) in the *GATA binding protein 4 (GATA4)* gene is linked to an increase risk in the likelihood to develop AUD (Treuitlein et al., 2009; Edenberg et al., 2010; Kiefer et al., 2011). GATA4 is one regulator of ANP transcription. Preclinical genetic assessment of rodents predisposed to high alcohol consumption has recapitulated the human genome findings of ANP association with increased alcohol consumption. In addition, different genotypes of GATA4 alter alcohol-cue induced reactivity (Jorde et al., 2014). Specifically, G-allele carriers of GATA4, which increases ANP levels, display significantly lower alcohol-cue-induced amygdaloidal activations compared to AA-homozygote carriers that are associated with lower ANP levels (Jorde et al., 2014).

The alcohol-preferring (P) rat is an established model of alcoholism (Bell et al., 2006, 2016). Microarray studies indicate that P rats have increased Npr1 (NPRA) and Npr2 (NPRB) gene expression levels in the Acb compared to NP rats as well as increased Npr1 in the VTA

(McBride et al., 2012, 2013). Mutagentically-derived mice lacking functional NPR-A systems displayed greater stress-induced alcohol consumption and enhanced levels of anxiety (Mutschler et al., 2010). Finally, a history of chronic alcohol consumption mediates the genetic expression of ANP. In abstinent AUD patients, mRNA-expression of ANP was increased while there was a corresponding decrease in the methylation of the ANP promoter region (Hillemacher et al., 2009).

Despite the convergent genetic indication of a potential role for ANP in regulating AUD, there are a limited number of preclinical studies that have investigated ANP involvement in AUD-related behaviors. Central administration of ANP can reduce alcohol withdrawal seizures in mice (Kovacs, 2000). There are some reports that acute and chronic moderate alcohol drinking increases ANP levels in plasma and the brain (Guillaume et al., 1996, 1997). Peripheral administration of ANP reduces alcohol withdrawal induced anxiety (von der Goltz et al., 2014). Taken together, these findings suggest ANP may regulate alcohol consummatory behaviors.

In humans, acute alcohol consumption elevates plasma ANP levels in healthy individuals (Gianoulakis et al., 1997). Increased ANP is also observed during early withdrawal and may be associated with delirium tremens (Kiefer et al., 2002; Kiefer and Wiedeman, 2004; Kovacs, 2003). In abstinent AUD patients, plasma levels of ANP are negatively correlated with anxiety, craving for alcohol, and perceived stress about not consuming alcohol (Koopman et al., 2014). Thus, clinical research has indicated a possible benefit of increased levels of ANP in treatment-seeking AUD patients.

The mesocorticolimbic (MCL) dopaminergic (DA) reward system mediates drug-reward and compulsive drug-seeking behaviors (Hauser et al., 2011, 2015; Koob and Volkow, 2016). ANP is present in the MCL DA system, including the nucleus accumbens (Acb) and ventral tegmental area (VTA; Langub et al., 1995; Mantyh et al., 1987; Quirion, 1988). In the rat, ethanol (EtOH) is directly self-administered into the Acb Shell (AcbSh) and posterior VTA (pVTA), but not the Acb Core (AcbCo) or anterior VTA (Engleman et al., 2009; Rodd-Henricks et al., 2000). The AcbSh and the pVTA also mediate EtOH-seeking behavior by P rats (Hauser et al., 2011, 2015). The objectives of the current study were to test the hypothesis that MCL-ANP activity mediates EtOH self-administration and -seeking behaviors in female P rats.

2. METHODS

2.1 Animals

Adult female P rats (postnatal day 90) from the 66^{th} – 73^{rd} generations, weighing 250–325g at the start of the experiment, were used in this study. Rats were maintained on a 12-hour reversed light-dark cycle with lights off at 0900. Food and water were available *ad libitum* throughout the experiment, except during operant testing. The animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the institutional animal care and use committee of the Indiana University School of Medicine and are in accordance with the guidelines of the Institutional

Animal Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the *Guide for the Care and Use of Laboratory Animals* (Research Institute for Laboratory Animal Research, 2011).

2.2 Chemical Agents and Vehicle

Ethyl alcohol (190 proof; McCormick Distilling Co., Weston, MO, USA) was diluted to 10%, 20%, and 30% (1st experiment) or 15% (2nd experiment) with reverse osmosis water for operant oral EtOH self-administration sessions in this study. ANP (rat; Millipore-Aldrich, St. Louis, MO, USA) was diluted with artificial cerebrospinal fluid (aCSF) to doses of 10 μg/0.5μl and 100 μg /0.5μl. The aCSF consisted of: 120.0 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM Mg SO_4, 25.0 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 10.0 mM D-glucose.

2.3 Operant Apparatus

The EtOH self-administration procedures were conducted in 3-lever experimental chambers (Coulbourn Instruments, Whitehall, PA, USA) contained within ventilated, sound-attenuated enclosures for the 1st experiment. The operant levers, located on the same wall, were 15 cm above a grid floor and 13 cm apart. A trough was directly beneath each lever, from which a dipper cup would raise to present fluid. Upon a reinforced response on the respective lever, a cue light was illuminated in the drinking trough and 4 seconds of dipper cup (0.1 ml) access was presented. On the opposite wall, water was available through a non-contingent spout connected to a water bottle that was placed outside of the chamber. The three-lever operant procedure was chosen for the first experiment because presentation of multiple concentrations of EtOH can increase the baseline levels of EtOH intake (Bell et al., 2014).

For the EtOH-seeking experiment, procedures were conducted in standard two-lever (15% EtOH, H_2O) operant chambers (Coulbourn Instruments, Whitehall, PA, USA) contained within ventilated, sound-attenuated enclosures.

2.4 Operant Training

Female P rats were placed into the operant chamber without prior training. Operant sessions were 60 minutes in duration and occurred 7 days each week for 10 weeks (described in Hauser et al., 2012, 2015). For three lever (10%, 20%, 30% EtOH) operant protocol, all levers were maintained on an FR1 schedule of reinforcement for 4 weeks. At the end of this time, the response requirement for EtOH was increased to an FR-3 schedule for 3 weeks, and then an FR-5 schedule for 3 weeks. For two lever operant protocol, EtOH (15%) levers were maintained on an FR1 schedule of reinforcement for 4 weeks. At the end of this time, the response requirement for EtOH was increased to an FR-3 schedule for 3 weeks, and then an FR-5 schedule for 3 weeks. The water lever was maintained on an FR-1 schedule.

2.5 Stereotaxic Surgeries

Bilateral stereotaxic implantation of 22-gauge guide cannulas (Plastic One, Roanoke, VA) was performed while rats were under isoflurane anesthesia (2%). Cannulas were aimed 1.0 mm above the AcbSh, AcbCo, the area ventral to the AcbSh, or the pVTA. Coordinates based on Paxinos and Watson (1998) were (a) AcbSh: −1.7 mm posterior to bregma, + 2.4

mm lateral to the midline, and −7.5 mm ventral; (b) AcbCo: −1.7 mm posterior to bregma, + 2.7 mm lateral to the midline, and −6.5 mm ventral; (c) area ventral to the AcbSh: −1.7 mm posterior to bregma, + 2.4 mm lateral to the midline, and −7.7 mm ventral; or (d) pVTA −5.8 mm posterior to bregma, + 2.1 mm lateral to the midline, and −8.5 mm ventral from the surface of the skull at an angle 10° from vertical (Fig. 1). A 28-gauge stylet was placed into the guide cannula extending 0.5 mm beyond the tip. After surgery, rats were individually housed, administered Carprofen 2–3 days post-surgery, and allowed to recover for 7 days. Animals were handled for at least 5 min daily beginning on the fourth recovery day.

2.6 Experiment 1: ANP in Nucleus Accumbens Shell, Core, and Ventral to Nucleus Accumbens Shell on Ethanol Self-Administration

Female P rats were placed in 3-lever operant chambers and given concurrent access to 10, 20, and 30% (v/v) EtOH with a water bottle available on the opposite side of the chamber, away from the operant levers. Multiple concentrations of EtOH were used in this experiment because the protocol increases EtOH consumption and it allows for the examination of tested compounds on the preference for specific concentrations of alcohol. For example, a compound may reduce the number of total EtOH responses, but not alter the amount of alcohol consumed, indicating a switch to preference of higher concentrations of alcohol. Following the 10th week of daily operant sessions, rats were implanted bilaterally with guide cannulae aimed at the AcbSh, AcbCo, or the area ventral to the AcbSh. On the day after surgery, rats were returned to the operant chambers. One week after surgery, all rats received a mock injection prior to placement in the operant chambers for 2 consecutive days to habituate rats to the procedures. Immediately prior to the next 7 consecutive operant sessions, rats (n = 5–10/group) were given bilateral injections of aCSF, 10 μg/0.5 μl ANP or 100 μg/0.5 μl. ANP was administered through 28-gauge injectors inserted bilaterally to a depth of 1.0 mm beyond the end of the guide cannulae connected to a Hamilton 25 μl syringe driven by a microinfusion syringe pump (Harvard Apparatus, MA, USA). A total volume of 0.5 μl was administered over a 30 sec period per side with the injector tip left in place for an additional 30 sec for diffusion. Following the 7 daily microinjection sessions, P rats were given 7 consecutive daily operant sessions without any drug administration.

2.7 Experiment 2: Effects of ANP in Posterior Ventral Tegmental Area on Pavlovian Spontaneous Recovery (PSR) of Ethanol-Seeking Behavior and Locomotor activity

Experimentally naïve rats received operant access to 15% EtOH (v/v) and water. After the 10th week of EtOH training, the rats underwent 7 days of extinction training with neither water nor EtOH available (Hauser et al., 2011, 2015). With the exception of no fluid being presented, the delivery system operated as the preceding EtOH self-administration sessions. A single concentration of EtOH was used in the current experiment to eliminate potential motivational differences (reward saliency/valance) from the data if subjects differ on seeking divergent concentrations of EtOH. After extinction training, all rats were maintained in the home cages for 14 days, i.e., no operant sessions. Surgery was performed after 7 days in the home cage. Following the home cage phase, rats were given 4 consecutive Pavlovian Spontaneous Recovery (PSR) test sessions (Hauser et al., 2015). The PSR test sessions were identical to the extinction protocol conditions. The FR5-FR1 (EtOH-water) schedule, lever contingencies and dipper functioning were maintained, but EtOH and water were absent. All

rats received 2 mock injections during the home-cage phase, prior to the infusion of ANP. Bilateral injections of aCSF, 10 μg/0.5 μl, or 100 μg/0.5 μl ANP (n = 7–9/group) were administered immediately prior to the 1st PSR session. ANP was microinjected using the same procedure as in Experiment 1.

The rats were home cage for a week following the PSR test. The locomotor activity (LMA) test was conducted the week after home cage. LMA recording sessions took place in plexiglass recording chambers (43.2 × 43.2 × 30.5 cm), which were each equipped with 16 infrared beam transmitters spanning the x and y axes of the field, plus another 8 transmitters on the z axis (Med Associates, Inc., St. Albans, VT). Transmitter and detector arrays for translational motion were located 5 cm from the chamber floor and spaced 2.5 cm apart. Data were collected using the Activity Monitor 5.0 software (Med Associates, Inc., St. Albans, VT). ANP was microinjected using the same procedure as in Experiment 1. Bilateral injections of aCSF, 10 μg/0.5 μl, or 100 μg/0.5 μl ANP (n = 7–9/group) were administered immediately prior to the 60 min LMA session.

2.8 Experiment 3: Effects of ANP in the Nucleus Accumbens Shell and Posterior Ventral Tegmental Area on Saccharin Self-Administration

Experimentally naïve female P rats received operant access to 0.0125% Saccharin and water daily for 8 (AcbSh) or 10 (pVTA) consecutive weeks. Rats were implanted bilaterally with guide cannulae aimed at the AcbSh or the pVTA. On the day after surgery, rats were returned to the operant chambers. One week after surgery, all rats received a mock injection prior to placement in the operant chambers for 2 consecutive days to habituate rats to the procedures.

For rats with guide cannulae implanted into the AcbSh (n = 5/group), rats received bilateral microinjections of aCSF, 10 μg/0.5 μl ANP or 100 μg/0.5 μl ANP immediately prior to the next 7 consecutive operant sessions. For rats with guide cannulae implanted into the pVTA (n = 8), all rats received bilateral microinjections of 100 μg/0.5 μl ANP immediately prior to the next 7 consecutive operant sessions. ANP was microinjected using the same procedure as in Experiment 1.

2.9 Statistical Analyses

Overall operant responding (60 minutes) data were analyzed with a mixed factorial ANOVA with a between subject factor of dose and a repeated measure of ‘session’. For the studies, the baseline measure for the factor of ‘session’ was the average number of responses on the EtOH lever for the 3 sessions immediately prior to surgery. Post-hoc Tukey’s b tests were performed to determine group differences. For the PSR experiments, the baseline measure for the factor of ‘session’ was the average number of responses on the EtOH lever for the last 3 extinction sessions. The Tukey’s b post-hoc comparison is a modified Tukey post-hoc comparison that reduces the prohibitive penalty for unequal sample size. The statistical integrity of ANOVAs is greatly reduced when the assumption of ‘Independence of Measure’ is violated (Kepple and Zeddeck, 1986). Therefore, within subject variables should be analyzed by post-hoc t-tests and orthogonal contrasts (Keppel and Zeddeck, 1986). We are reporting only the findings from t-test analyses to ease interpretation of the data. We adhere

to the replicated finding that Type 1 error rate inflations is Ψ_i ($i = 1, –, J – 1$). Given the effect size of the current data set, Type 1 error rate inflation is not a concern for any reported analyses (cf., Rodgers and Roberts, 2013; Roberts, 2011).

3. RESULTS

3.1 Experiment 1: Effects of ANP in the Nucleus Accumbens Shell, Core, or Ventral to Nucleus Accumbens Shell on Ethanol Self-Administration

The overall analysis revealed a significant 'injector location' x 'ANP Dose' x 'EtOH concentration' x 'Session' interaction term ($F_{(64,214)} = 1.8$, $p = 0.003$). The significant 4-way interaction term was decomposed by holding 'injection location' constant (performing 3-way mixed factor ANOVAs for each 'injection location'). In P rats with injections placed within the AcbSh (Fig. 2), the 'Sessions' x 'ANP Dose' and 'EtOH concentration' interaction terms were significant (p-values < 0.014). The significant 3-way interaction term was further decomposed by holding 'ANP Dose' constant (determining the effects of ANP on EtOH self-administration at each dose level). Microinjection of aCSF (Fig. 2, top panel) did not alter EtOH self-administration compared to baseline ($p = 0.71$). The statistical analysis revealed that for P rats microinjected with 10 or 100 μg ANP into the AcbSh, there were significant 2-way interactions terms of 'Session' x 'EtOH Concentration' (p values < 0.02). Planned within subject comparisons examining the number of level responses for a particular concentration of EtOH between injection days and baseline levels indicated that in P rats microinjection of 10 μg ANP (middle panel) into the AcbSh reduced self-administration of 20% during injection days 3 and 7 and 30% EtOH during injection days 1, 2, 4, 5, and 6. Planned within subject comparisons for P rats receiving microinjection of 100 μg ANP into the AcbSh (bottom panel) indicated that self-administration for 10% EtOH was reduced for all 7 injection days, the self-administration for 20% EtOH was reduced during injection days 3, 4, 5, and 7, and the self-administration for 30% EtOH was reduced during injection days 2–7 (within subjects comparisons – $p < 0.05$).

In P rats with injectors placed within the AcbCo (Fig. 3), there was a significant effect of 'Session' [$F_{(7,7)} = 5.6$, $p = 0.0181$] and 'EtOH concentration' [$F_{(2,12)} = 4.0$, $p = 0.048$], but no other significant findings (p-values > 0.43). Further analysis of the 'EtOH concentration' effect, revealed that, in all microinjection groups, responding for the 10% EtOH solution was reduced significantly, but this was compensated by an increase in responding for the 30% EtOH. Microinjection of ANP (or aCSF) into areas ventral to the AcbSh (Fig. 4) did not alter EtOH self-administration (p-values > 0.09).

The use of multiple EtOH concentrations has a number of benefits, but a distraction is the complexity of the visual representation of the data. To assist in the understanding of the effects of microinjections of ANP into the AcbSh, AcbCo, and areas ventral to the AcbSh we have converted lever responses into an estimated g/kg intake for each subject for each session (Fig. 5). Given that the dependent measures are estimates of EtOH intake, we performed a simple 3-way analysis with between subject factors of 'ANP Dose' and 'injector location' and a within subject variable of 'Session'. The mixed factor ANOVA indicated that there was a significant 3-way interaction term ($p = 0.014$). There were no significant 2-way interaction effects (p values > 0.25) for rats receiving microinjections of

ANP into the AcbCo (Fig. 5; top panel) or areas ventral to the AcbSh (bottom panel). Only in rats receiving microinjection of ANP into the AcbSh was there a significant 'ANP Dose' x 'Session' interaction term ($p = 0.007$). Individual ANOVAs performed on each 'Session' contrasting 'ANP Dose' indicated that during injection days 2, 3, 6, and 7 microinjections of 10 or 100 μg ANP reduced estimated EtOH intake compared to aCSF controls (Tukey's b). During injection days 4 and 5, the three injections groups significantly diverged for the estimated EtOH intake (Tukey's b). Although blood EtOH levels were not collected in the current study, previous studies have shown that during 1-hour sessions P rats can achieve pharmacological relevant blood EtOH concentrations (50–80 mg%; Bell et al., 2016, McBride et al., 2014; Hauser et al., 2012).

3.2 Experiment 2: Effects of ANP in the Posterior Ventral Tegmental Area on Ethanol-Lever Responding in the PSR Test and Locomotor activity

Analysis of the number of responses on the lever previously associated with the delivery of EtOH for rats microinjected with aCSF, 10 μg ANP, or 100 μg ANP (Fig. 6A) indicated a significant effect of 'session' [$F_{(4,19)} = 0.821$; $p < 0.001$], 'dose' [$F_{(2, 22)} = 4.353$; $p = 0.026$], and 'session' by 'dose' interaction [$F_{(8,40)} = 0.654$; $p = 0.031$]. Post-hoc comparisons indicated that P rats given 10 μg ANP or 100 μg ANP responded significantly less than P rats given aCSF. Pair-wise t-test comparisons showed that the aCSF group responded significantly more during the 1st PSR session compared to the extinction baseline, whereas there were no differences for the 10 or 100 μg ANP groups during the 1st PSR session compared to extinction baselines. There were no significant differences for water lever-responding ($p \geq 0.08$; Fig 6B). Pair-wise t-test comparisons showed that the 10 μg ANP group responded significantly more during the 1st PSR session compared to the water extinction baseline, whereas there were no differences for aCSF or 100 μg groups during the 1st PSR session compared to extinction baseline (Fig. 6B).

The effects of microinjection of aCSF, 10 μg, or 100 μg ANP into the pVTA on general locomotor activity of P rats were also examined (Fig. 7). Microinjection of aCSF, 10 μg, or 100 μg ANP had no significant effect on general locomotor activity. The overall analysis conducted was a mixed factor ANOVA with between subject variable of 'ANP Dose' and within subject variable of 'Time Block'. There was no significant interaction term or main effect of 'ANP Dose' (p values > 0.12). There was a significant main effect of 'Time Block', but this was the result of all groups being more active during the initial period of the LMA test session. An additional analysis was performed on overall LMA (ANOVA) with a between subject factor of 'ANP Dose'. The results indicated no significant effect of 'ANP Dose' on LMA ($p = 0.07$).

3.3 Experiment 3: Effects of ANP in the Nucleus Accumbens Shell and Posterior Ventral Tegmental Area on Saccharin Self-Administration

There were two separate analyses performed. For rats receiving microinjections into the AcbSh, a Mixed Factor ANOVA with the between subject factor of ANP Dose and the within subject factor of Session was performed on the number of Saccharin Lever Responses (Fig. 8; top panel). The overall analysis indicated that there was no significant ANP Dose x Session interaction term ($F_{16,214} = 0.46$; $p = 0.61$) or main effects of ANP Dose ($F_{2,13} =$

1.14; p = 0.57) or Session ($F_{8,76}$ = 0.72; p = 0.33). For rats receiving microinjections of ANP into the pVTA, a repeated measure ANOVA on the variable Session was performed. The analysis indicated that there was no significant effect of Session ($F_{6,14}$ = 0.42; p = 0.68).

3.4 Statistical Assessment for Violations of Assumptions of ANOVAs

The assessment for normally distributed dependent variables (normalcy) for all analyses performed was determined by the Shapiro-Wilks test. Testing for homogeneity of variance was performed by the Levene's test. Sphericity of the data sets was determined through the Mauchly's (W) sphericity test and the traditional Greenhouse-Geisser assessment (and possible correction). Residual analysis for all data sets was performed by the OLS regression estimate for stochastic error. For all data sets presented, the Shapiro-Wilks tests indicated normal distributions except for water level responding during Experiment 2 (skewness to zero). Levene's tests were non-significant for all analyses (p values > 0.09), indicating that there was no evidence of heterogeneity of variances in the data sets. Similarly, tests for sphericity on all data sets failed to detect a violation of this assumption of the ANOVA procedure (p values > 0.51). The OLS regression analysis indicated little evidence for random or unpredictability within group dispersion from the mean for Experiment 1 and 3. In contrast, OLS regression analysis indicated that there were group differences in stochastic error for EtOH-seeking in Experiment 2 (p = 0.028).

Specifically, for rats microinjected with 10 or 100 μg ANP into the pVTA there was no significant residual stochastic estimate (p values > 0.85; indicating randomness in residuals). In contrast, OLS regression analysis in rats receiving aCSF microinjections into the pVTA prior to EtOH-seeking testing indicated that there was a predicted residual from the mean during PSR1 and Ext Base (p = 0.03). This result indicates that responding during the last 3 days of extinction training (Ext Base) influenced the observed (compared to predicted) residual from the mean during PSR1. Simply stated, there appears to be a correlation between residuals (not a correlation in responding) in this group for Ext Base and PSR1.

4. DISCUSSION

The major findings of this study are that local infusion of ANP into AcbSh reduced EtOH self-administration (Figs. 2 and 5) and local infusion of ANP into the pVTA reduced context-induced EtOH-seeking (Fig. 6) in female P rats. The data indicate that activation of ANP receptors within the mesolimbic DA pathway mediates multiple EtOH self-administration related behaviors.

The current findings add to a slowly growing literature indicating consistent effects of ANP activation on alcohol-related behaviors. NPR-A knockout mice had greater EtOH intake following repeated swim stress and stress aggravated neurobehavioral EtOH-withdrawal symptoms (Mutschler et al., 2010). Similar results have indicated that ANP regulates EtOH intake through mitigation of anxiety-like processes. The potential beneficial anxiolytic effects of ANP is indicated by reports demonstrating that peripheral administration of ANP reduces alcohol-withdrawal induced anxiety (von der Goltz et al., 2014).

The current study suggests that the application of 10 μg of ANP into the AcbSh reduced EtOH self-administration for higher concentrations of EtOH (20% or 30%) over 7 days but did not alter responding for a lower EtOH concentration (10%). The highest dose of ANP (100 μg) had a more robust effect and reduced EtOH self-administration of all concentrations (greater than a 73% reduction for all 3 levers) on days 3–5 and 7, whereas the 100 μg dose of ANP reduced EtOH self-administration of the low EtOH concentration (10%) or for both low (10%) and high (30%) EtOH concentrations, on the 1st, 2nd, and 6th days respectively (Fig. 2). The 100 μg dose of ANP also greatly reduced the estimated g/kg/session of EtOH intake levels (Fig. 5). ANP effects within the AcbSh were specific to EtOH self-administration because ANP within the AcbSh did not alter saccharin self-administration (Fig. 8). These findings extend previous ANP research showing that microinfusion of ANP into the AcbSh is involved in regulating EtOH self-administration that is not associated with withdrawal-related behaviors, and that repeated exposure to the highest dose of ANP is more effective in attenuating overall EtOH self-administration than a low dose of ANP into the AcbSh.

In contrast, ANP infused into the AcbCo (Fig. 3) or ventral to the AcbSh (Fig. 4) did not alter EtOH self-administration. These findings indicate that ANP has region specific effects within Acb and are comparable with previous studies that showed the AcbSh and AcbCo have a differential involvement in mediating EtOH reward (Engleman et al., 2009; Ding et al., 2014) and EtOH-seeking behaviors (Hauser et al., 2015). Previous reports indicated that the AcbSh, but not AcbCo, supports the reinforcing actions of EtOH (Engleman et al., 2009), and that activation of D_1 receptors within the AcbSh, but not the AcbCo, is required to maintain the reinforcing actions of EtOH within the pVTA (Ding et al., 2014).

ANP has also been shown to mediate alcohol craving. Drug craving is a motivational state for drug-seeking behavior and it is thought to be the biological precursor to drug relapse (Sinha, 2013). A human study found that during the first 2 weeks of abstinence, AUD patients had lower ANP plasma levels and reported more intense and frequent craving along with more anxiety. Additionally, higher ANP plasma levels were associated with a decreased risk for alcohol relapse during the 3 months pharmacological treatment period (Kiefer et al., 2002, 2011; Kiefer and Wiedeman, 2004). Other research indicates lower levels of ANP are positively correlated with alterations in ANP-promoter DNA methylation and negatively correlated with the level of craving for alcohol (Hillemacher et al., 2009; Glahn et al., 2014; Hodes and Lichtstein, 2014).

The present study used the PSR context-induced EtOH-seeking model (Rodd et al., 2004) to examine the effects of ANP within the pVTA on the expression of EtOH-seeking behavior. Previous reports demonstrated that activation of pVTA DA neurons was needed for PSR expression of EtOH-seeking behavior (Hauser et al., 2011). Microinfusion of ANP into the pVTA inhibited responding on the EtOH lever during the PSR test, with both 10 μg and 100 μg doses of ANP having a robust attenuating effect (Fig. 6A). This effect was not due to general decreases in motor activity because water lever responses were either similar to or higher than the control groups (Fig. 6B). Moreover, ANP microinfused into pVTA did not alter motor activity in the LMA test (Fig 7). These findings indicate that ANP activity in the pVTA attenuated the PSR expression of EtOH-seeking behaviors in female P rats, which

suggests ANP within the MCL reward pathway mediates, in part, alcohol craving-like behaviors. To our knowledge, this is the first study that has examined the effects of ANP on alcohol addiction behaviors in female rodents; therefore, additional research is also needed to determine if there are any sex differences of ANP effects on EtOH intake and EtOH-seeking behaviors.

Radioimmunoassay (Kawata et al., 1985; Quirion, 1988; Skofitsch et al., 1985; Zamir et al., 1986) and in situ hybridization (Langub et al., 1995) studies confirm the presence of ANP in the Acb and VTA. The current manuscript is the first to report the effects of ANP in the MCL DA reward areas of the Acb and pVTA on EtOH self-administration and EtOH seeking behaviors, respectively. As it was previously mentioned, the gene expression of Npr1 (NPRA) has been shown in Acb and VTA (McBride et al., 2012, 2013) and Npr2 (NPRB) in the VTA in P rats (McBride et al., 2013), however, it is unclear if ANP is acting on neurons or glia cells or fibers in VTA and Acb. However, previous studies have demonstrated that ANP may act on the DA system. Intracereobroventricular (ICV) injection of ANP can decrease DA levels within the hypothalamus and hippocampus (Nakao et al., 1986). Furthermore, ANP has the ability to induce increases in tyrosine hydroxylase (TH) activity through cGMP/PKG dependent mechanisms, resulting in increased catecholamine (DA) synthesis (Takekoshi et al., 2000). Moreover, peripheral administration of haloperidol, a dopamine D2 receptor antagonist, can block ICV ANP anxiolytic effects (Biro et al., 1995; Bhattacharya et al., 1996) as well as block the ability of ANP to increase consolidation of memory in both active and passive avoidance paradigms (Bidzseranova et al., 1991a,b). The ability of haloperidol to modulate the central effects of ANP suggests that ANP may be acting to modulate DA neurotransmission. Taken together, these studies suggest that ANP effects on EtOH-self administration and EtOH-seeking in the current study may involve regulating DA.

Genetic alterations in ANP activity is another possible explanation for the current findings. Interestingly, GWAS studies found that alcohol dependence is associated with the gene GATA4, a transcription factor that regulates ANP (Edenberg et al., 2010; Karpyak et al., 2014; Treutlein et al., 2009). These authors found that GATA4 influences ANP plasma concentration. The AA/AG genotype of SNP rs13273672 may be indicative of diminished metabolic responsiveness and consequently reduced ANP synthesis. GATA4 was also shown to be associated with increased relapse rates in AUD patients (Kiefer et al., 2011). Collectively, these studies suggest that reduced functioning of the ANP system may contribute to the development of AUDs.

Neuroimmune dysregulation is implicated in alcohol addiction behaviors (Roberto et al., 2018; Erickson et al., 2019). ANP has anti-inflammatory properties and neuroprotective properties (De Vito, 2014). It can inhibit the activation of the proinflammatory transcription factor nuclear factor kappa B (NF-kB) and reduce the synthesis and release of proinflammatory cytokines and chemokines [i.e., interleukin 1 beta (IL-1b), IL-6, and tumor necrosis factor-alpha (TNF-alpha), as well as monocyte chemoattractant protein-1 (MCP-1)] (De Vito, 2014). In addition, ANP has been reported to reduce anxiety-like behavior (Bhattacharya et al., 1996; Meyer and Herrmann-Lingen, 2017). The ability of ANP to

regulate EtOH consumption and EtOH-seeking may, in part, be through its regulation of the neuroimmune systems, as well as its anxiolytic effects.

In conclusion, the ability of local applications of ANP into the AcbSh and pVTA to attenuate EtOH-self administration and EtOH-seeking behavior, respectively, suggests that these subregions of the MCL reward pathway mediate, at least in part, inhibition of alcohol-associated behaviors. Recent clinical evidence has suggested that the ANP system is a candidate for the development of pharmacotherapeutics for the treatment of depression, anxiety, post-traumatic stress disorder, and addictive behaviors (Marazziti et al., 2020). Further studies are needed to determine neuronal, neurochemical, and molecular mechanisms that modulate ANP activity within the MCL in the pursuit of novel pharmacotherapies targeting subsets of the AUD population. However, a concluding caveat should be considered. Although the current experiments examined the effects of ANP within discrete brain regions with little diffusion of ANP offsite, this is not a practical treatment paradigm in humans. The well-established cardiac effects of ANP may make the compound an impractical valid treatment of AUD.

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HIGHLIGHTS

- ANP into nucleus accumbens (Acb) shell reduced ethanol (EtOH) intake
- ANP into the Acb core and ventral to Acb did not alter EtOH intake
- ANP into posterior ventral tegmental area reduced EtOH-seeking
- ANP within mesolimbic dopamine (DA) system is involved in mediating EtOH-associated behaviors

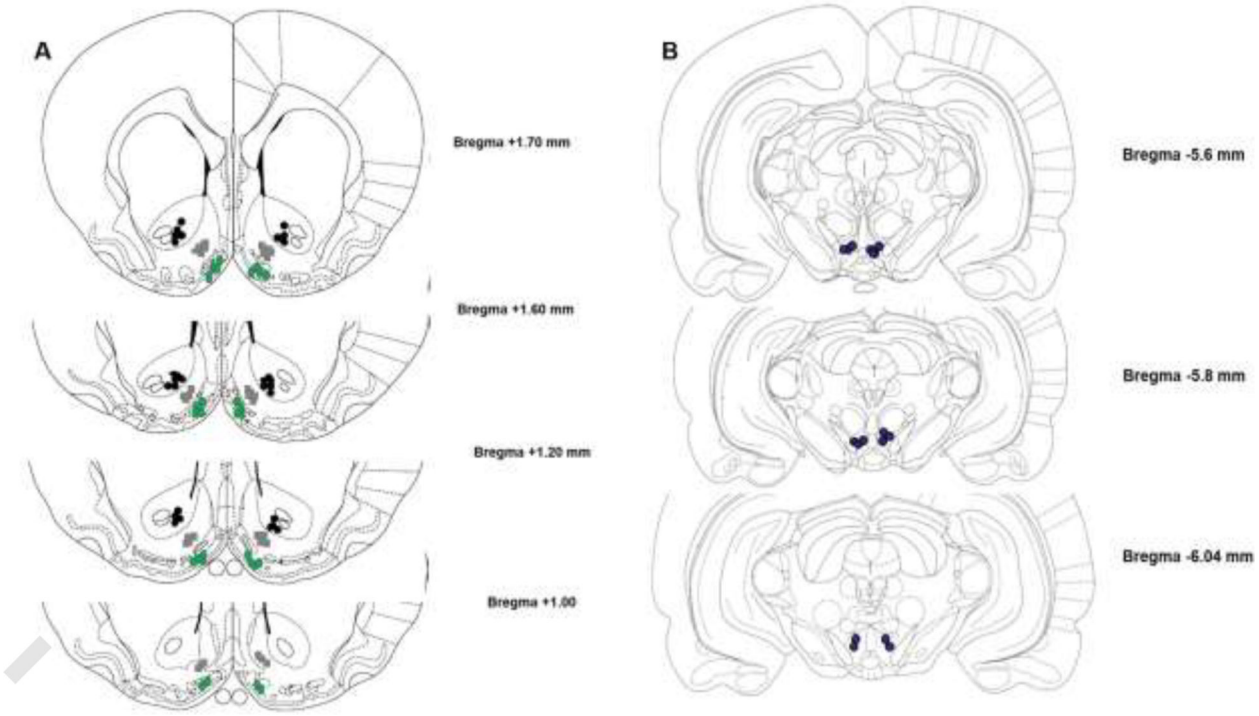

Fig. 1:

Representative placements for the microinjections of aCSF and ANP into the AcbSh, AcbCo, ventral to the AcbSh, and pVTA of adult female P rats are shown. (A) Grey squares represent placements of injection sites within the AcbSh, black circles represent placements of injection sites within the AcbCo, and green hexagon represents placements of injection sites within the area ventral to the AcbSh (defined as +1.7 to + 1.0 mm bregma). (B) blue circles represent placements of injection sites within the pVTA (defined as −5.6 to − 6.04 mm bregma).

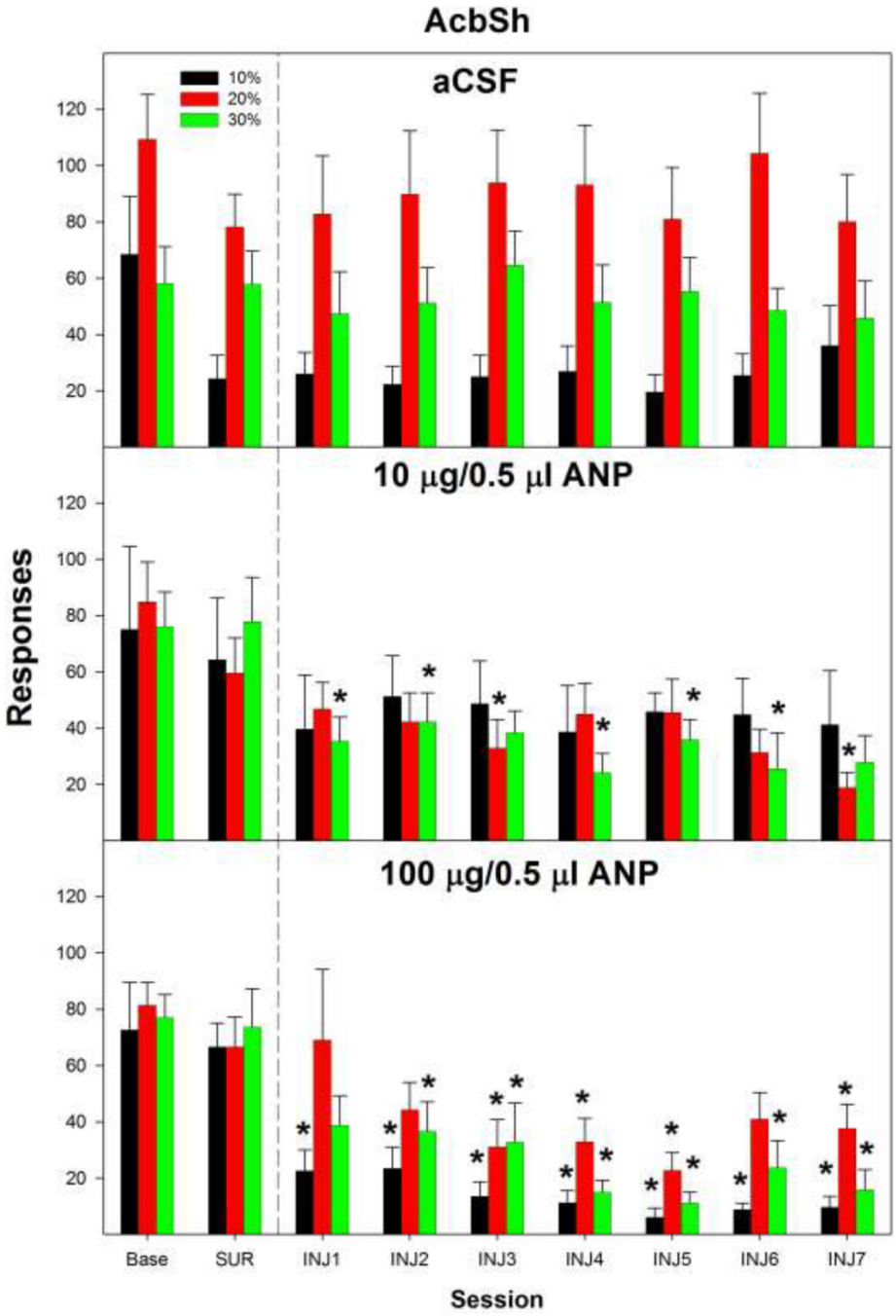

Fig. 2:

Mean (± S.E.M.) responses per session on the levers associated with the delivery of 10%, 20 % and 30 % EtOH (concurrent access) by P rats given aCSF (n = 5), 10 µg/0.5 µl (n=10), or 100 µg/0.5 µl (n = 10) of ANP into the AcbSh prior to their operant sessions. Asterisk (*) indicates that rats administered 10 µg/0.5 µl or 100 µg/0.5 µl of ANP significantly ($p < 0.05$) reduced their responses on the EtOH lever compared to baseline (Base) levels. SUR = last 3 days post-surgery.

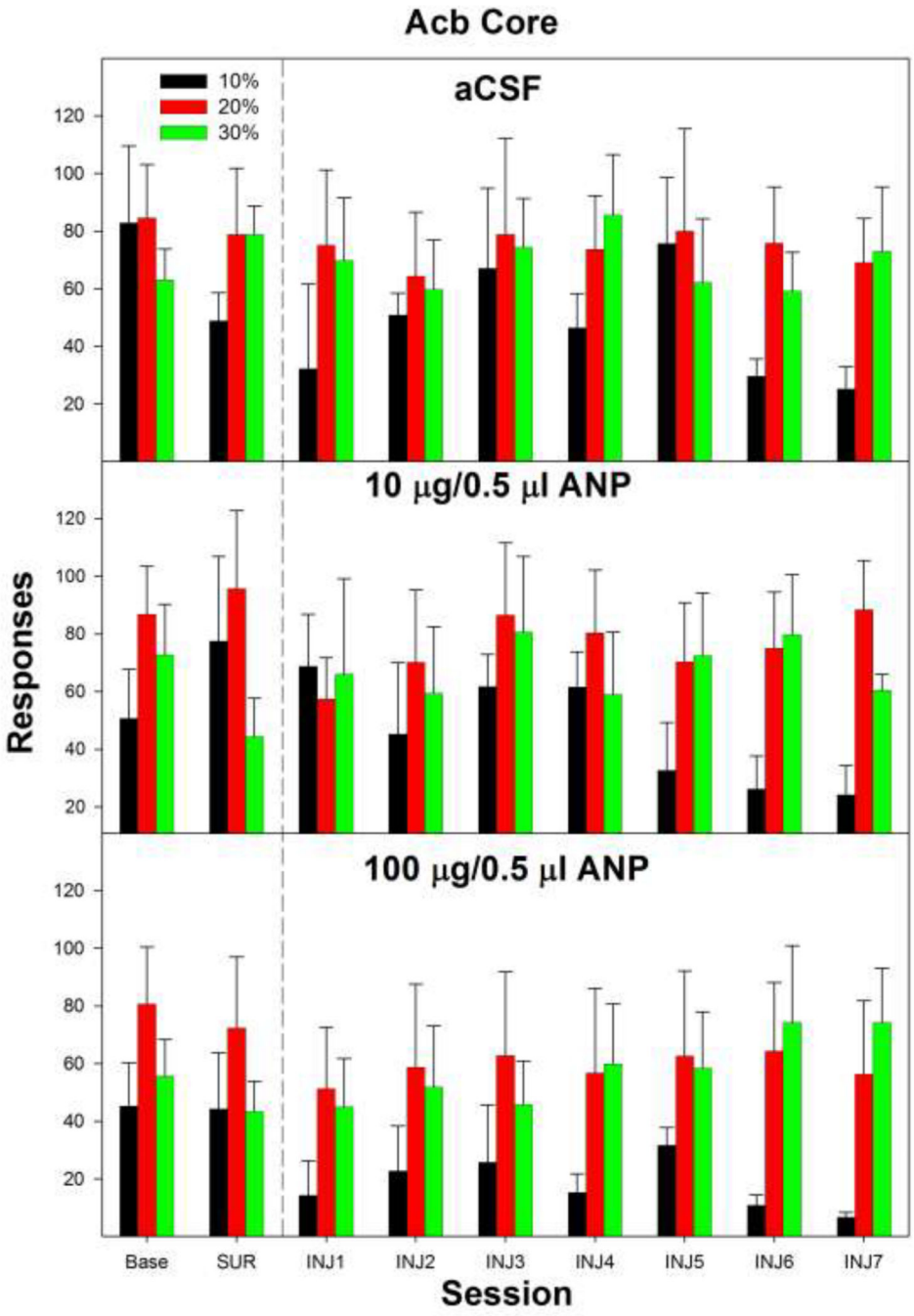

Fig. 3:

Mean (± S.E.M.) responses per session on the levers associated with the delivery of 10%, 20 % and 30 % EtOH (concurrent access) by P rats given aCSF (n = 5), 10 µg/0.5 µl (n=10), or 100 µg/0.5 µl (n = 10) of ANP into the AcbCo prior to their operant sessions. There were no significant differences among the 3 groups. Base = last 3 days EtOH training and SUR = last 3 days post-surgery.

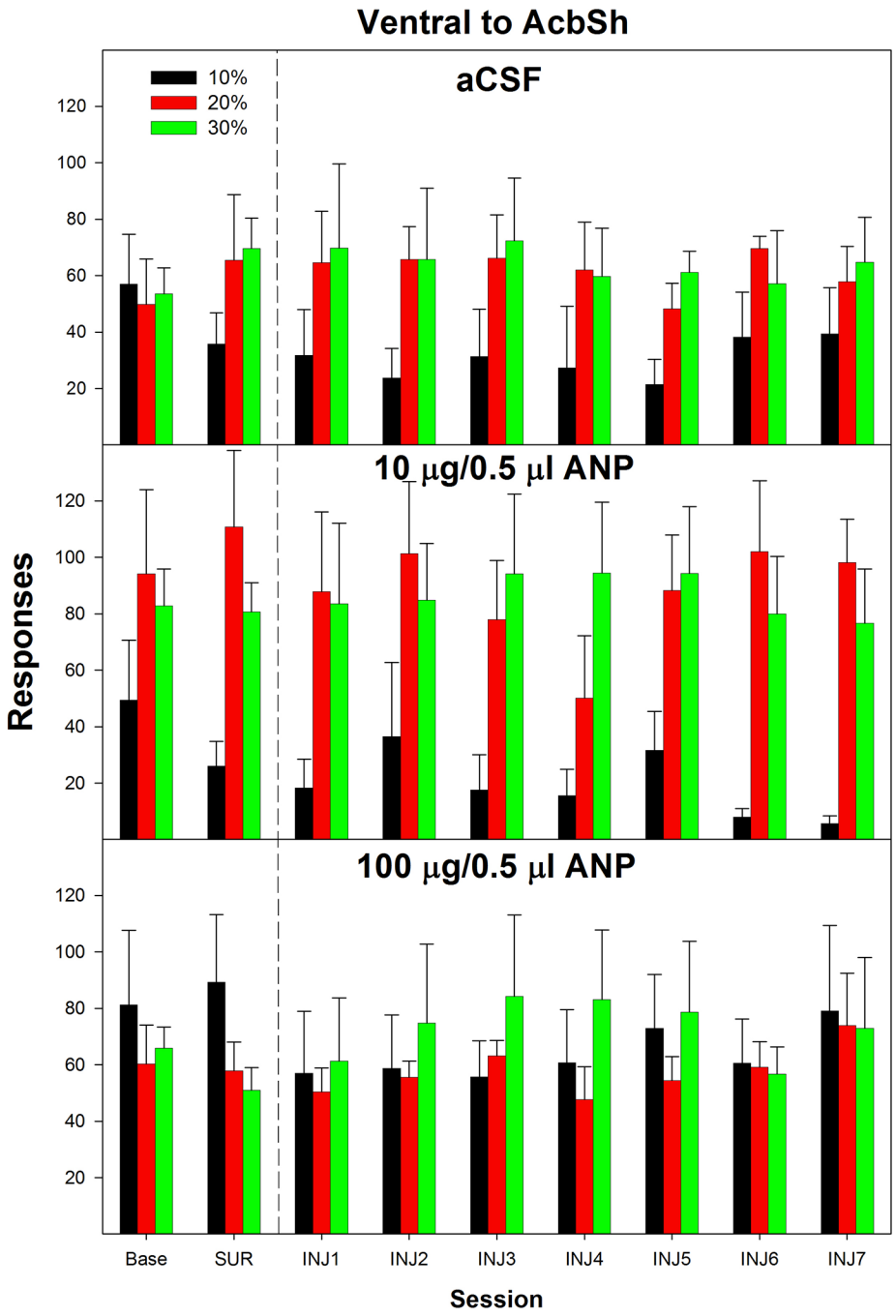

Fig. 4.

Mean (± S.E.M.) responses per session on the levers associated with the delivery of 10%, 20 % and 30 % EtOH (concurrent access) by P rats given aCSF (n = 5), 10 µg/0.5 µl (n=10), or 100 µg/0.5 µl (n = 10) of ANP into the ventral to the AcbSh prior to their operant sessions. There were no significant differences among the 3 groups. Base = last 3 days EtOH training and SUR = last 3 days post-surgery.

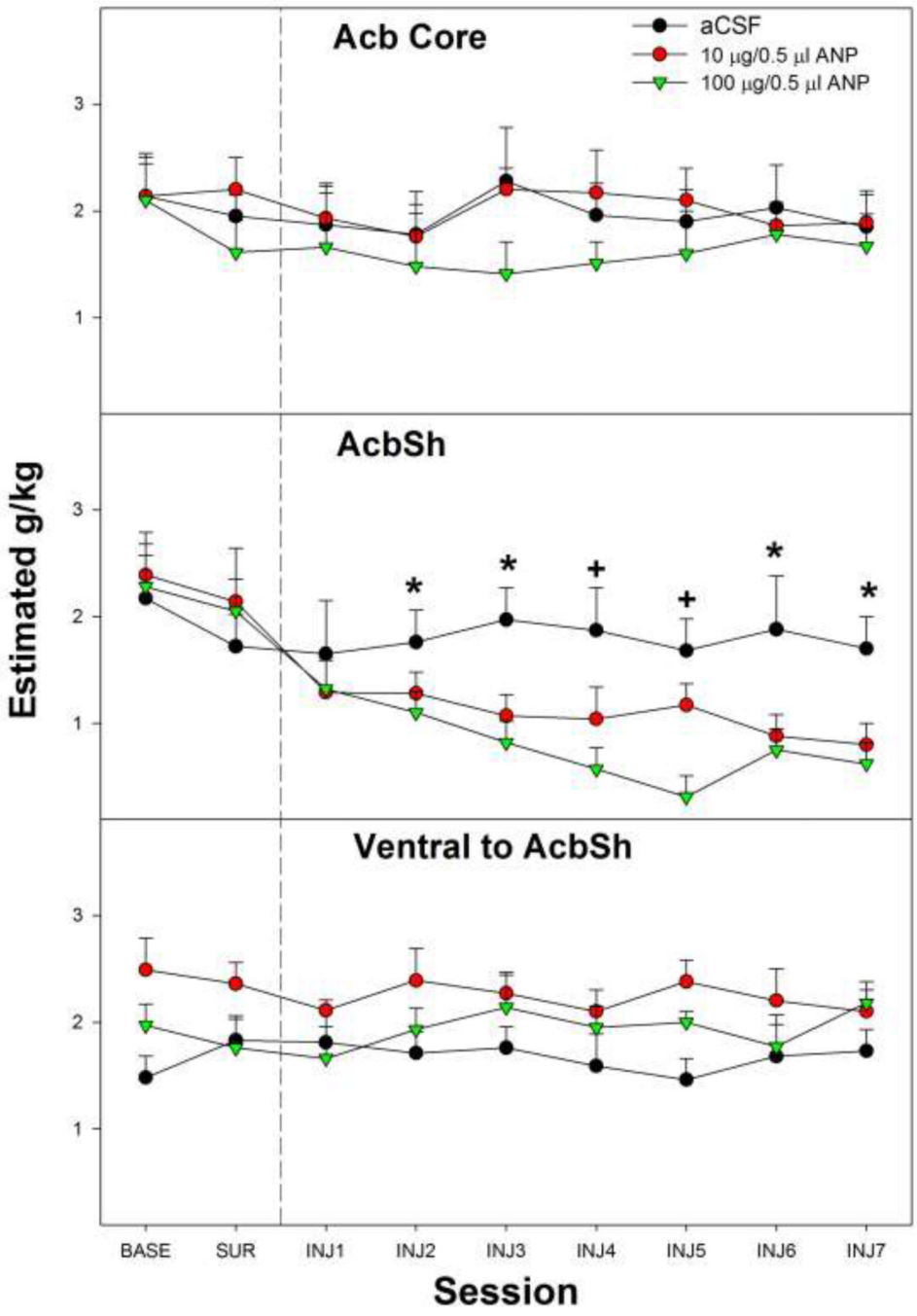

Fig. 5:

Mean (± S.E.M.) total estimated EtOH intake (g/kg) per session associated with the delivery of EtOH by P rats given aCSF (n = 5), 10 µg /0.5 µl (n=10), or 100 µg/0.5 µl (n = 10) of ANP into the AcbSh, AcbCo, or ventral to AcbSh prior to their operant sessions. Asterisk (*) indicates that rats administered 10 µg /0.5 µ1 and 100 µg/ 0.5 µl of ANP significantly ($p < 0.05$) reduced estimated EtOH intake compared to aCSF controls. Plus (+) indicates that rats administered 10 µg /0.5 µ1 and 100 µg/ µl of ANP significantly ($p < 0.05$) reduced estimated EtOH intake compared to aCSF controls, and 100 µg/ µl of ANP significantly ($p <$

0.05) reduced estimated EtOH intake compared to 10 μg /0.5 μ1. Base = last 3 days EtOH training and SUR = last 3 days post-surgery.

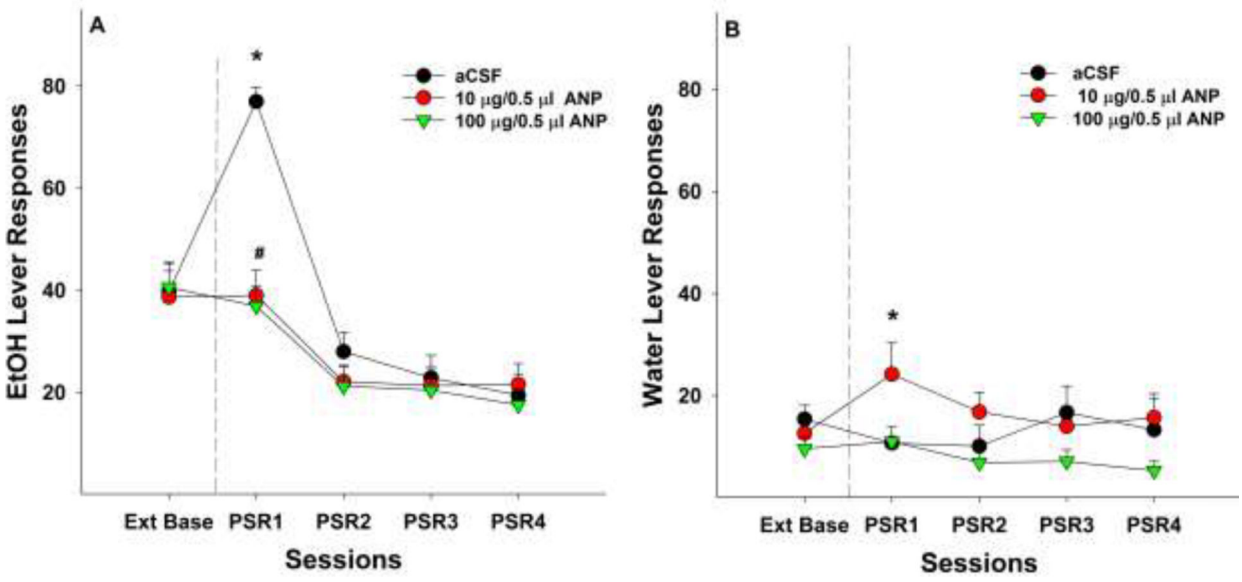

Fig. 6:

Mean (± S.E.M.) responses per session on the lever previously associated with the delivery of 15% EtOH (A) or water (B) by P rats given aCSF (n = 7), 10 µg/0.5 µl (n = 9), or 100 µg/0.5 µl (n = 9) of ANP into the pVTA prior to only the 1st PSR session. (A) Asterisk (*) indicates that rats given 10 µg/0.5 µl or 100 µg/0.5 µl of ANP responded significantly ($p < 0.05$) less on the EtOH-associated lever during the 1st PSR session compared to aCSF and the aCSF group responded significantly ($p < 0.05$) more on the EtOH-associated lever during the 1st PSR session compared to extinction baseline (Ext Base) levels. (B) Asterisk (*) indicates that rats given 10 µg/0.5 µl of ANP responded significantly ($p < 0.05$) more on the water-associated lever during the 1st PSR session compared to extinction baseline levels.

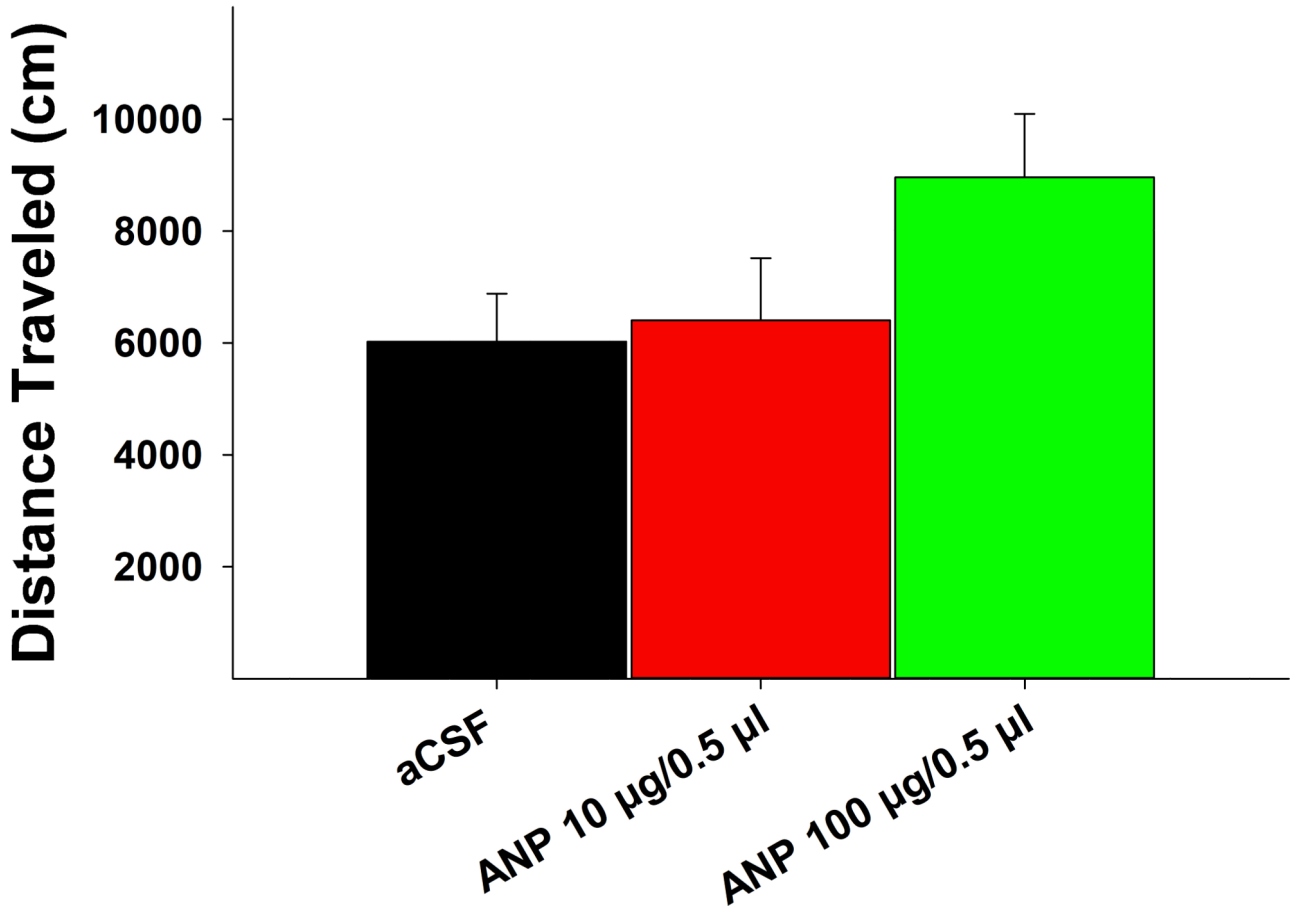

Fig. 7:

Mean (± S.E.M.) distance traveled in the LMA test. P rats were given aCSF (n = 7), 10 µg/0.5 µl (n = 9), or 100 µg/0.5 µl (n = 9) of ANP into the pVTA prior to the LMA test. There were no significant differences in LMA between the groups.

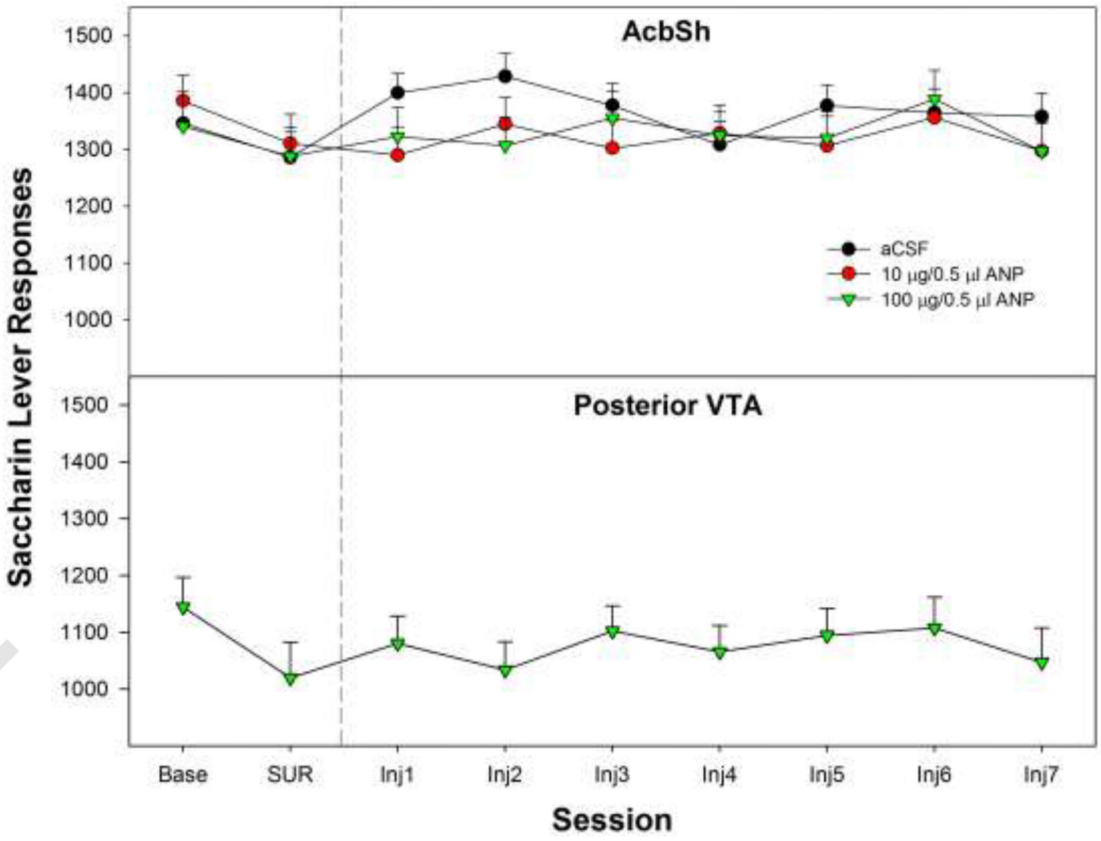

Fig. 8:

Mean (± S.E.M.) responses per session on the lever associated with the delivery of 0.0125% saccharin by P rats given aCSF (n = 5), 10 µg/0.5 µl (n= 5), or 100 µg/0.5 µl (n = 5) of ANP into the AcbSh or P rats given 100 µg/0.5 µl (n = 8) of ANP into pVTA prior to their operant sessions. There were no significant differences between groups for AcbSh. Base = last 3 days EtOH training and SUR = last 3 days post-surgery.