

AWARD NUMBER: W81XWH-13-1-0126

TITLE: Stress and PTSD Mechanisms as Targets for Pharmacotherapy of Alcohol Abuse, Addiction and Relapse

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REPORT DATE: DEC 2018

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
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1. REPORT DATE DEC 2018		2. REPORT TYPE Final		3. DATES COVERED 30 Sep 2013 - 29 Sep 2018	
4. TITLE AND SUBTITLE Stress and PTSD Mechanisms as Targets for Pharmacotherapy of Alcohol Abuse, Addiction and Relapse				5a. CONTRACT NUMBER W81XWH-13-1-0126	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dennis Rasmussen, PhD E-Mail: drasmuss@u.washington.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Seattle Institute for Biomedical and Clinical Research 1660 S Columbian Way #151F Seattle, WA 98108-1532				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We have demonstrated that (1) alcohol-naïve rats exhibiting high acoustic startle response (associated with increased anxiety-like behavior) develop increased subsequent alcohol intake and preference which are correlated with acoustic startle response determined before initial alcohol access, providing a prospective index of vulnerability to developing alcohol abuse, as well as insights into mechanism; (2) suppression of noradrenergic signaling decreases alcohol drinking in rats with a history of traumatic stress, but not in rats without this stress history (informing clinical studies in which subjects exhibit variable responses to prazosin); (3) this treatment also suppresses alcohol drinking by rats with history of compulsive-like alcohol drinking, but increases alcohol drinking by rats that do not (these results inform clinical studies in which subjects have been reported to exhibit opposite responses that are dependent on family history of compulsive alcohol drinking; (4) suppression of noradrenergic signaling at the time of traumatic stress decreases acquisition of increased voluntary alcohol drinking long after the stress, which provides a new model for preventive treatment. Accomplishment 1 has been published, 2-4 are in preparation for publication. Animal work is complete. There have been no changes in scope, but some delays due to personnel issues and methods refinements. Additional data analyses will likely reveal additional stress-PTSD-alcohol interactions.					
15. SUBJECT TERMS PTSD, alcohol, ethanol, prazosin, noradrenergic, startle, anxiety, stress, pharmacotherapy, prevention, rat, abuse					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 46	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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1. **INTRODUCTION:** Studies from our research group demonstrating that the well-characterized, safe, well-tolerated and FDA-approved α 1-adrenergic receptor antagonist (AR), prazosin, is effective not only in treating combat post-traumatic stress disorder (PTSD) symptoms but in decreasing alcohol drinking in both human and rat studies provide much-needed breakthroughs in the development of effective pharmacotherapies for alcohol use disorders as well as for PTSD. However, much work remained to determine conditions in which this treatment to reduce noradrenergic hyperactivation will be effective, characteristics of individuals who are most likely to respond, and underlying mechanisms providing bases for additional treatments. Our immediate objective was to identify key variables in rat models that will inform and complement human studies, providing a powerful translational approach for most efficiently and rapidly developing and implementing effective new pharmacotherapies for alcohol use disorders and co-morbid PTSD.
2. **KEYWORDS:** alcohol, ethanol, PTSD, prazosin, noradrenergic, startle, anxiety, stress, pharmacotherapy, prevention, rat, abuse
3. **OVERALL PROJECT SUMMARY:** There were no significant changes in the project goals or studies planned.
 - **OBJECTIVES** There were 3 objectives of this project, which remained unchanged. To be consistent with the organization and section headings of the current SOW, these 3 major objectives continue to be presented here as SPECIFIC AIMS 1-3, although each of these specific aims now also are identified (in parentheses) as Objective 1, 2 or 3, for further clarity. Also consistent with the organization of the current SOW, Tasks 1-6 are discussed within these three SPECIFIC AIMS (Objectives), identified by bold text.
 - **SPECIFIC AIM 1 (Objective 1): Determine relationship of hyperexcitability, anxiety and α 1-adrenergic receptor-mediated signaling to excessive voluntary alcohol drinking**, *providing information from rat models that will likely reveal especially promising bases for:*
 - a) *Prospectively identifying subsets of individuals who are highly vulnerable to developing alcohol use disorders (AUDs).* **(TASK 1)**

STATUS: We have demonstrated that alcohol-naïve rats exhibiting high acoustic startle response (which is associated with increased anxiety-like behavior) develop increased subsequent alcohol intake and alcohol preference in an intermittent alcohol access (IAA) paradigm. These results are consistent with the central hypothesis for all other studies in this research project, i.e., that hyper-responsiveness characteristic of PTSD, alcohol withdrawal/abstinence, and increased noradrenergic activation contributes to – or at least is associated with – development of increased alcohol drinking. This work has been completed and the published paper is included in the appendix (Rasmussen and Kincaid,

2015). *b) predicting who is most likely to respond to prazosin with decreased alcohol drinking. (This work was not assigned a separate specific TASK # in the SOW, but it is a component of several of the proposed experiments. To facilitate discussion, we include it here as TASK 1b).* STATUS: Although our initial work suggests that high acoustic startle and increased anxiety-like behavior is associated with increased suppression of alcohol drinking in response to prazosin, as hypothesized, we are investigating responses in all alcohol access and PTSD-like conditions of this overall investigation, as planned. Consequently, resolution of this issue was not planned to be finalized until all experiments would be completed, and remains under analysis (see "Changes, Problems and Actions to Resolve Them" in Section 3. As described in the original proposal, prazosin was administered prior to voluntary alcohol drinking in rats that had been previously characterized for acoustic startle and anxiety-like behaviors in the differing experimental models used in these studies. In an initial trial with the rat PTSD model used throughout these investigations (and described in detail in the proposal), 51 young male Wistar rats received either 10 seconds of inescapable footshock (traumatic shock; TS) or no shock (NS) followed by 4 weekly contextual reminders (R) of the TS or NS (but no further application of TS or NS). The rats were then provided 4 weeks of intermittent alcohol access (IAA, 24 h/day free choice between 20% alcohol vs water on 3 non-consecutive days/week) to establish stable elevated levels of voluntary episodic alcohol drinking. Alcohol access was then further restricted to 1 hour on each IAA day for the next 4 weeks. On 1 day of each week, each rat received an intra-peritoneal (IP) injection of either vehicle alone (VEH), the alpha-1 adrenergic receptor antagonist, prazosin (PRZ, 1.5 mg/kg), the beta-adrenergic receptor antagonist, propranolol (PROP, 5 mg/kg), or the combination of PRZ+PROP (1.5 mg/kg, 5 mg/kg) at 30 min before the 1 hour alcohol access period, with each rat receiving each of the 4 treatments in counterbalanced order over the 4 weeks. In the rats that had not received TS ("Non-Stress" in the figure) there was not a significant change in alcohol intake in response to either drug alone or to the combination of drugs (**Fig. 1**), even though the doses of each drug were the same as we have previously demonstrated to consistently decrease alcohol drinking in selectively-bred alcohol-preferring (P) rats derived from the Wistar strain. In

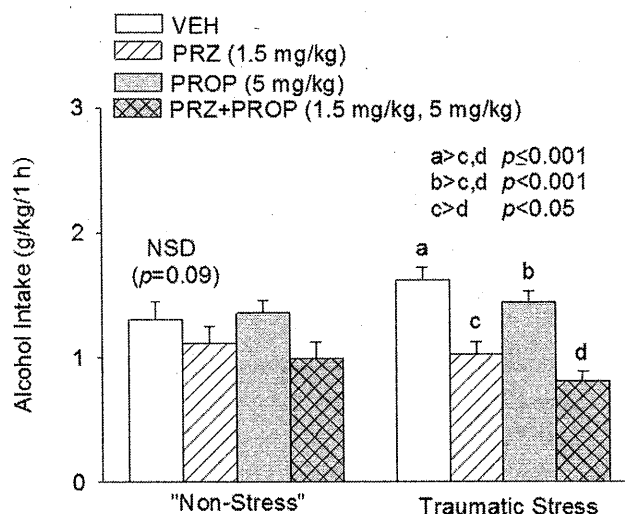


FIG. 1

contrast, rats that had received a single application of TS at least 8 weeks before testing ("Traumatic Stress" in the figure) exhibited consistently decreased acute alcohol intake in response to prazosin treatment, and this suppression was enhanced by combining the prazosin treatment with propranolol treatment (this enhanced suppression of alcohol drinking by addition of propranolol to prazosin treatment is consistent with our previous results in P rats). These results suggest that, in normal outbred rats voluntarily drinking alcohol in an IAA model, the response to reduction of noradrenergic activity by prazosin or prazosin+propranolol is dependent on history of previous stress.

Due to the high potential clinical significance of these results, we then repeated this entire experiment with another set of rats, again starting with the PTSD model followed by 4 weeks intermittent alcohol access and then this same acute drug testing. The results are shown in **Fig. 2**. In this repeat experiment, prazosin or prazosin+propranolol again each reduced alcohol drinking in rats with a history of single traumatic stress 13 weeks earlier but – again – not in rats that had received non-stress treatment. Together, these two complementary experiments now provide consistent evidence that a single 10 second traumatic stress with occasional brief contextual reminders produced conditions in which prazosin or prazosin+propranolol more effectively reduced alcohol drinking long after an acutely traumatic experience.

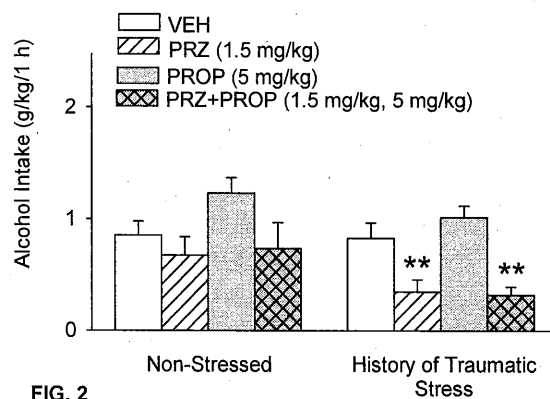


FIG. 2

Our finding that effectiveness of prazosin or prazosin+propranolol in suppressing alcohol drinking is dependent on preceding history of traumatic stress will likely have great impact in informing further clinical trials and applications of prazosin treatments, since clinical responses to prazosin treatment have been inconsistent. Further characterization of relationships of this response to rat PTSD-related behavioral predictive indices will increase this utility and impact. In addition, this rat model may provide an especially effective preclinical model for further resolving the brain, neuroendocrine and behavioral mechanisms mediating this disparity in responses.

We recently presented this work at the 2017 Annual Meeting of the Research Society on Alcoholism, in a symposium entitled "The Neglected Catecholamine: Noradrenergic Mechanisms in Regulation of Alcohol-Related Behaviors". Dr. Rasmussen was Co-Organizer of this symposium. His research presentation, entitled "Variability in alcohol drinking responses to suppression of noradrenergic signaling in outbred

rats" also included additional new results from a study employing long-term [IAA vs continuous access] model of compulsive alcohol drinking that is discussed later in this report. This study is in preparation for publication in collaboration with Murray Raskind, MD.

c) preventing initial acquisition of AUDs in prospectively-identified vulnerable individuals. (TASK 2)

STATUS: The central hypothesis of this experiment was that rats exhibiting high acoustic startle before the initiation of IAA would subsequently exhibit high IAA alcohol intake (as we have demonstrated in Task 1), and that continuous treatment to suppress noradrenergic signaling before and throughout IAA would prevent this acquisition of high alcohol drinking. This experiment thus required continuous treatment of the rats during introduction of IAA. As discussed in detail in the previous (Year 3) report, we had repeated problems with several approaches to providing this continuous treatment. We had originally proposed to accomplish this with intraperitoneal administrations 3 times each day, which proved to be highly stressful within only a few days. We then instead conducted an entire study using implantable osmotic minipumps to maintain prolonged (4 week) constant administration (with prior approval at the time of the first annual report). Figures presenting results were included in the previous progress report (Year 3). In short, there were two major problems. The first was that osmotic pumps delivering only vehicle suppressed alcohol intake compared to untreated rats, suggesting that either stress associated with the implanted pump or perhaps the administration of vehicle alone suppressed alcohol drinking. The second was that prazosin or [prazosin+propranolol] were ineffective in consistently altering the alcohol drinking. This lack of response was eventually determined to be due to the fact that the prazosin had precipitated out of solution within the prazosin and [prazosin+propranolol] pumps, clogging the orifice through which the drug is delivered. Consequently, as proposed in the Year 3 report, we repeated this study using an oral route of administration. In order to circumvent the known problem of prazosin short metabolic half-life that requires multiple daily treatments over the entire 4 week treatment period, we proposed in the year 3 report to instead use the drug doxazosin, an analog of prazosin which is functionally essentially identical - with the same alpha-1 adrenergic receptor antagonist specificity - but which has a much longer half-life that allows once/daily administration in clinical applications. We had already reported that doxazosin suppresses alcohol drinking in rats comparably to prazosin, and doxazosin had been reported (by a group including my colleague and consultant, Dr. Murray Raskind) to suppress clinical symptoms of PTSD similarly to prazosin. To allow investigation of multiple doxazosin dosing (as originally proposed for prazosin), we proposed (in the Year 3 report) to investigate only VEH vs doxazosin treatments. So, although the proposed study was completed, we then adjusted the model to eliminate problems that we had encountered and to repeat the study

again, this time with doxazosin administered in pieces of flavored gelatin which the rats find to be palatable, even when containing dissolved doxazosin. The results are shown in **Figs. 3 - 5**. The treatment groups are Vehicle (flavored gelatin alone), 1 Doxazosin (5 mg/kg doxazosin administered once, at start of dark period), and 2 Doxazosin (5 mg/kg doxazosin administered twice; once at the start of the dark period and again near the end of the dark period). As illustrated in **Fig. 3**, administration of doxazosin 1x/day produced a small reduction in the development of IAA alcohol drinking, and 2X/day produced a marked suppression of this initial development of increased alcohol drinking. **Fig. 4** illustrates that daily treatment with doxazosin likewise suppressed development of increased alcohol preference (relative to water). **Fig. 5** illustrates that 1x/day doxazosin appears to be potentially most effective in suppressing development of increased IAA alcohol drinking in the subset of rats that had the lowest acoustic startle response (measured here as Vmax) characterized before the start of alcohol access, although variance in the results is high and the sample size is inadequate for a robust test.

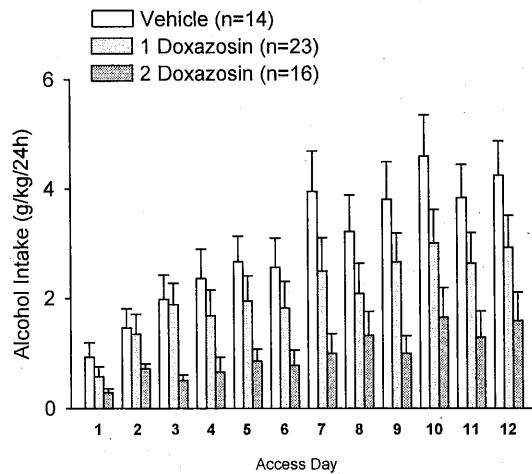


FIG. 3

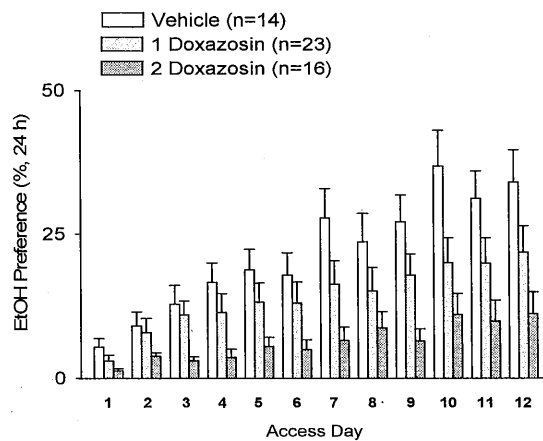


FIG. 4

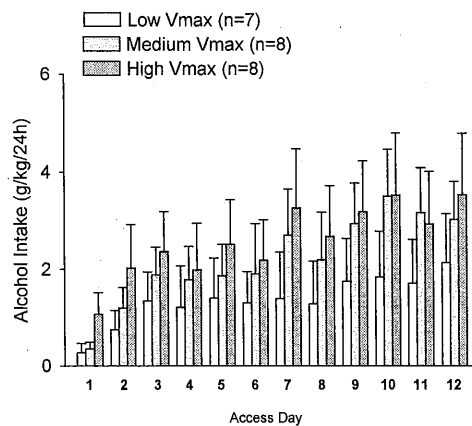


FIG. 5

d) predicting who is most vulnerable to progression from

voluntary to compulsive alcohol drinking, and determining whether this “compulsive-like” alcohol drinking is responsive to doxazosin (doxazosin was substituted for prazosin in the Year 3 Annual Report). This study also addresses whether alcohol drinking remains up-regulated long after this history of compulsive drinking, and whether this “compulsive-like” alcohol drinking remains responsive to doxazosin. **(TASK 3)**

STATUS: This prolonged (28 week) study evaluated the hypothesis that prazosin would suppress high alcohol drinking even in rats that had exhibited high acoustic startle and associated anxiety-like behavior before IAA and which subsequently develop compulsive alcohol drinking (i.e., alcohol drinking that is maintained even after a distasteful adulterant – e.g., quinine - is added to the alcohol). As we recently reported at the 2017 Annual Meeting of the Research Society on Alcoholism symposium organized by the PI, entitled “The Neglected Catecholamine: Noradrenergic Mechanisms in Regulation of Alcohol-Related Behaviors”, rats that received alcohol in the IAA model for 24 weeks developed markedly elevated levels of alcohol drinking that was compulsive-like (i.e., it was resistant to adulteration of the alcohol with bitter tasting quinine) whereas rats that received continuous access to alcohol did not develop elevated levels of consumption and the consumption was not compulsive-like. Furthermore, the compulsive-like alcohol drinking of the IAA rats was suppressed by doxazosin treatment, whereas non-compulsive alcohol drinking in the rats receiving continuous alcohol access was conversely increased by doxazosin treatment (**Fig. 6**). These results were surprising, but consistent with results of a recent clinical study in the laboratory of Lorenzo Leggio, MD PhD (which were also presented in our symposium at the 2017 Research Society on Alcoholism meeting) - i.e., Dr Leggio demonstrated that alcoholic subjects who are family history positive (+) for alcoholism continue to drink high levels of alcohol even in the clinical trial setting (i.e., they exhibit evidence

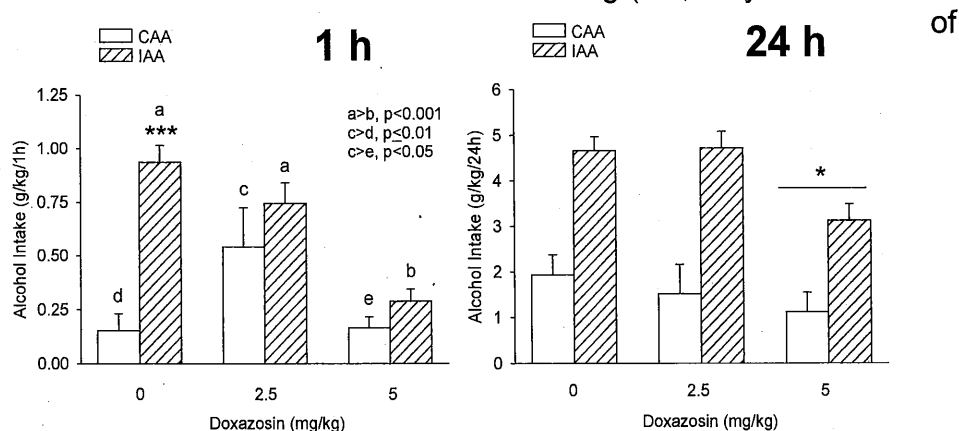


FIG. 6

compulsive drinking) and also respond to doxazosin with decreased alcohol drinking, whereas alcoholic subjects who are family history (-) exhibit markedly decreased alcohol drinking in the clinical trial setting (i.e.,

the clinical setting produces a large placebo effect consistent with non-compulsive drinking) but doxazosin paradoxically increases this alcohol drinking. The surprising results with our rat model thus are remarkably consistent with clinical evaluations with alcoholic subjects. Furthermore, our results in this study complemented results in our study described above in Task 1b (and also presented in this same 2017 symposium) demonstrating that although prazosin administration to rats that had received prior traumatic stress produced decreased alcohol drinking, prazosin administration to rats that had not received prior traumatic stress did not. As highlighted by the Discussant/Question Moderator for the symposium, Dr. Raye Litten of the National Institute for Alcohol Abuse and Alcoholism (NIAAA), these results strongly suggest that a subset of subjects who are predisposed to develop compulsive alcohol drinking due to genetics, prolonged severe alcohol abuse, and/or traumatic stress history may be the subset of otherwise difficult-to-treat subjects most appropriate for treatment to reduce noradrenergic signaling with prazosin or doxazosin.

We are currently further analyzing whether initial acoustic startle response and behavioral characterization can predict the subsequent development of compulsive-like drinking and also the response to prazosin or doxazosin. The manuscript describing these studies will then be finalized and published.

SPECIFIC AIM 2 (Objective 2): Evaluate PTSD/alcohol interactions, *providing information from rat models that will likely reveal especially promising bases for:*

a) determining cause-effect between AUDs and vulnerability to developing PTSD, i.e., does PTSD increase vulnerability to developing AUDs and do AUDs increase vulnerability to developing PTSD?. (TASK 4) STATUS: This work compares production of a PTSD-like behavioral and acoustic startle profile in rats with vs without a previous recent history of alcohol liquid diet-induced excessive prolonged alcohol intake. This work is nearly complete (see discussions of CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM in the Year 2 and 3 Annual Reports). We have already published a demonstration that symptoms in our rat PTSD model predict subsequent significantly increased development of excessive alcohol drinking, and we have further confirmed in a study reported at the 2016 Annual Meeting of the Society for Research on Alcoholism that PTSD in our rat model increased alcohol drinking. We are now conducting data analyses for the entire proposed investigation. A second part of this investigation is to determine whether a history of chronic high alcohol consumption and withdrawals increases vulnerability to develop PTSD following a traumatic stress (TS) during abstinence. In the completed experiment addressing this issue, provision of liquid diet containing alcohol for 3 cycles of 5 days alcohol, interrupted by 2 days of isocaloric control diet following cycles 1 and 2 (compared to

19 days of continuous control diet) produced increased acoustic startle after more than one month of imposed abstinence (**Fig. 7**; EtOH+NS), consistent with our previous work demonstrating that chronic alcohol liquid diet-induced dependence produced increased acoustic startle after 1 month of imposed abstinence, together with other PTSD-like neuroendocrine and behavioral symptoms. A single 10 sec TS after the 19 days of control diet (i.e., no alcohol exposure) followed by 4 weekly reminders of the stress (i.e., our rPTSD model) likewise increased acoustic startle (**Fig. 7**; PF+TS) consistent with our previous demonstration that this rPTSD model produces increased acoustic startle.

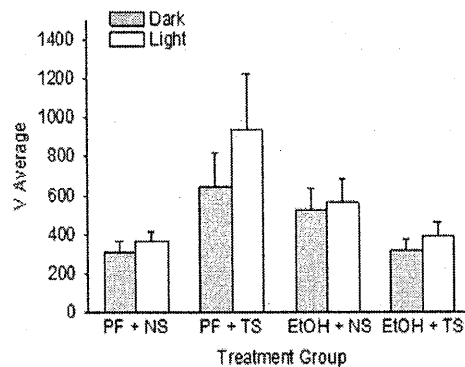


FIG. 7

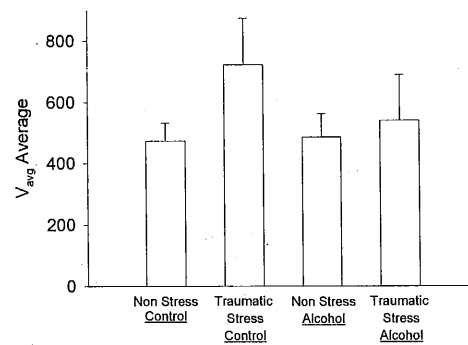


FIG. 8

However, when 3 cycles of 5 days of alcohol consumption were followed by traumatic stress applied 5 hours after removal of the alcohol diet followed by 4 weekly reminders, acoustic startle response was not increased (compared to rats receiving control diet without TS) (**Fig. 7**; EtOH+TS). This unexpected result led us to consider that perhaps rats receiving the alcohol liquid diet were still experiencing the pharmacologic effects of the alcohol even 5 hours after removal of the alcohol-containing diet, presumably due to slow stomach emptying of the high fat and high sugar liquid diet (resulting in relatively sustained delivery of alcohol) and slow clearance of the high dose of alcohol. If so, then the paradoxical lack of response to TS may have been due to sustained sedating and anxiolytic alcohol actions at the time of the TS, so that the TS was relatively non-traumatic. Consequently, we found it necessary to conduct another (unplanned) experiment, this time presenting the single TS during imposed abstinence one week after termination of the chronic alcohol consumption, rather than the 5 hours used in the study our protocol was modeled after, at a time that that we have confirmed plasma alcohol to have been completely cleared. In this new study with much delayed administration of the TS vs NS, ASR tested after 4 weekly reminders again was increased in the pair-fed control rats that had received TS (relative to pair-fed control rats that had received NS) but, surprisingly, chronic consumption of alcohol liquid diet that was terminated one week

before the TS again appeared to block the subsequent expected increase in ASR, as illustrated by the ASR during bright light exposure in **Fig. 8**.

So, although this experiment has now been conducted twice, and the experiments have provided similar results under the two different conditions of alcohol diet followed by TS, the interpretation is unclear. One possibility is that the liquid diet model may not have produced sufficiently robust alcohol dependence coupled with increased anxiety like behavior prior to the TS. Consequently, we found it necessary to conduct another (unplanned) experiment, this time presenting the single TS during imposed abstinence one week after termination of the chronic alcohol consumption, at a time that we have confirmed plasma alcohol to have been completely cleared and increased anxiety to persist. This additional trial has been completed and analysis is underway. Upon completion (see discussion of CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM), the overall results of this investigation will be published).

b) predicting who, among individuals with PTSD, is especially vulnerable to developing AUDs (TASK 5).

STATUS: This work addresses whether a rat PTSD-like behavioral and acoustic startle profile predicts subsequent acquisition of increased IAA alcohol intake. This work corresponds to **Task 5**. As discussed in the years 2 Annual Report, this work was conducted in parallel with Task 6 (discussed below), because the methods and time schedules were compatible, with the major difference being that the study addressing Task 6 also incorporated pharmacologic treatment to decrease noradrenergic signaling at the time of traumatic stress. The animal work is now complete, and the analyses and preparation for publication are underway (see discussion of CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM).

c) predicting who, among individuals with PTSD, is likely to respond to prazosin with decreased alcohol drinking.

STATUS: This work addresses whether a rat PTSD-like behavioral and acoustic startle profile predicts subsequent effectiveness of prazosin treatment in suppressing IAA alcohol intake. **As discussed in the years 2 and 3 Annual Reports, this work was not identified as a separate specific Task, but it is a component of several of the proposed experiments which were not all originally projected to be completed until the end of the overall project.** As discussed in the years 2 and 3 Annual Reports, this work was not scheduled as a separate specific task with a single proposed completion date, but it is a component of several of the proposed experiments which were not all originally projected to be completed until the end of the overall project. The animal work is complete, but the extensive analyses from several of the experiments are still underway (see discussion of CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM). Note that - as discussed in Task 1b - alcohol drinking, which commonly is increased in individuals with PTSD as

well as in our rat model of PTSD, was acutely suppressed by prazosin or prazosin+propranolol treatment in rats that had previously received traumatic stress and developed other rat PTSD symptoms, but not in rats that had not received traumatic stress 2 months earlier (presented these results as part of a 2017 Annual Meeting of the Research Society on Alcoholism symposium organized by the PI, entitled "The Neglected Catecholamine: Noradrenergic Mechanisms in Regulation of Alcohol-Related Behaviors"), and that – also discussed in Task 1b - we are evaluating this response in the context of other rat PTSD symptom characterizations which we have determined. Upon completion, this investigation will be published.

- **SPECIFIC AIM 3 (Objective 3): Determine whether the reduction of α 1-AR mediated signaling at the time of traumatic stress will prevent the subsequent development of increased alcohol abuse and PTSD, informing whether prophylactic prazosin treatment is likely to decrease vulnerability to PTSD and alcohol use disorders (TASK 6).**

STATUS: This work includes pharmacologic reduction of noradrenergic signaling at the time of traumatic stress to determine whether this treatment blocks subsequent development of a rat PTSD-like behavioral and acoustic startle profile, as well as increased subsequent IAA alcohol intake. As discussed in the year 3 Annual Report, we expanded this study to evaluate not only prazosin but also prazosin+propranolol treatments, due to recent results in some of our unrelated studies (funded by a separate NIH grant). We presented initial results at the 2016 annual meeting of the Research Society on Alcoholism which demonstrated that prazosin+propranolol treatment at the time of traumatic stress significantly decreased development of alcohol drinking long (8-12 weeks) after the traumatic stress. While analyzing the corresponding extensive acoustic startle and behavioral tests as discussed in the original proposal, addressing potential correlations and predictive validity of characterizations before and after the rat PTSD model in determining development of a PTSD-like condition and/or increased alcohol intake, it became apparent that an additional cohort of animals would facilitate more robust analyses of these behavioral characterizations and interactions. This additional animal work has been completed and final data analyses are underway. Results of trials with 141 male Wistar rats are described here. A new post-doctoral fellow (Rebecca Hendrickson, MD, PhD) is now helping to write up these results for publication (see discussion of CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM).

- The rats received a single traumatic stress (TS, 10 sec inescapable footshock) or nonstress (NS, 10 sec exposure to shock environment, but without administration of shock) followed by weekly contextual reminders (R) of the TS or NS, as described in detail in the original proposal. Either prazosin (1.5 mg/kg), prazosin (1.5 mg/kg) + propranolol (5 mg/kg), or vehicle alone were administered by

intraperitoneal (IP) injection 30-45 minutes before the TS or NS and again at 2 hours after the TS or NS. After 4-5 weekly R followed by 3-4 additional weeks of behavioral testing, the rats were allowed to voluntarily drink alcohol in an IAA model (20% ethanol vs water 2-bottle choice access for 24 hours/day on 3 non-consecutive days/week, as discussed in detail in the original grant proposal) for a total of 12 IAA trials (i.e., 3 IAA trials/week for 4 weeks). Alcohol intake was determined in the first 1 hour as well as in all 24 hours of each IAA session. The results for the first hour of drinking (thought to reflect motivation to drink alcohol for its acute pharmacologic effects, rather than drinking for caloric content and other factors affecting 24 h

drinking) and for all 24 hours of each IAA session are shown in **Fig. 9**. Alcohol intake in the first hour as well as in all 24 hours of each IAA trial increased gradually but irregularly in the 12 successive IAA trials ($p < 0.001$ by 2-way ANOVA with repeated measures on IAA trial), consistent with the gradual increase in alcohol drinking previously reported for the IAA model. A relatively consistent

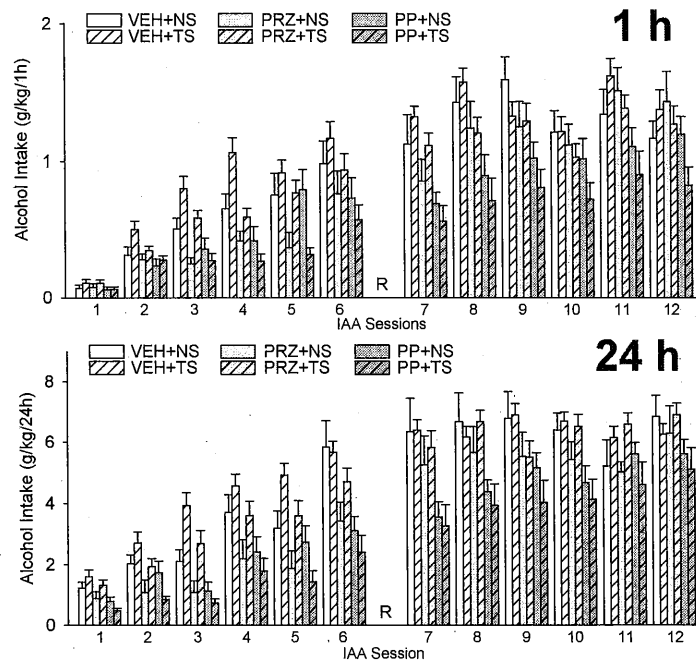


FIG. 9

treatment response pattern in the first 6 IAA sessions (i.e., the first 2 weeks, when alcohol intake was progressively increasing) was qualitatively different from the consistently elevated alcohol intake and consistent response pattern in IAA 7-14 (i.e., weeks 3-4). Consequently, subsequent analyses were then conducted after averaging data for each rat over sessions 1-6 and 7-14. **Fig. 10** illustrates suppression of IAA alcohol intake and alcohol preference by prazosin (PRZ) or prazosin+propranolol (PRZ+PRO) treatment only at the time of the single TS/NS exposure 8-12 weeks earlier.

Fig 10, Row 1: Alcohol intake during the first hour of each IAA, averaged across IAA trials 1-6 (LEFT PANEL) and 7-12 (RIGHT PANEL). In IAA trials 1-6 (LEFT PANEL), 1 h alcohol intake in rats that had received non-stress treatment (NS) 8 weeks prior to initiation of IAA was not significantly different among rats that had received VEH vs PRZ vs PRZ+PRO treatment at the time of the NS treatment. TS treatment 8

weeks prior to initiation of IAA increased 1 h alcohol intake ($p < 0.05$ vs NS) in rats that had been treated with VEH or PRZ at the time of the TS exposure. The 1 h alcohol intake in rats that had received TS was suppressed by PRZ treatment at the time of TS ($p < 0.05$ vs VEH), and further suppressed by PRZ+PRO treatment ($p < 0.01$ vs PRZ alone). In IAA trials 7-12 (RIGHT PANEL), average alcohol drinking during the first 1 h of each IAA was suppressed by PRZ+PRO ($p < 0.001$ vs VEH, $p = 0.01$ vs PRZ) treatment at the time of the single TS/NS exposure, independent of TS/NS exposure.

Each bar represents data from 15-35 rats. A preliminary study demonstrated that PRO (5 mg/kg) at the time of TS did not suppress (vs VEH) either 1 h or 24 h alcohol intake during IAA initiated 8 weeks later (data not shown).

Fig. 10, Row 2:
Alcohol intake during the entire 24 hours of each IAA, averaged across IAA trials 1-6 (LEFT PANEL) and 7-12 (RIGHT PANEL).

Average 24 h alcohol intake during IAA trials 1-6 (LEFT PANEL) was suppressed by PRZ treatment ($p < 0.05$ vs VEH) - and further suppressed by PRZ+PRO treatment ($p < 0.05$ vs PRZ, $p < 0.001$ vs VEH) - at the time of the single TS/NS exposure 8 weeks earlier, independent of TS vs NS exposure. In IAA trials 7-12 (RIGHT PANEL), 24 h alcohol intake was

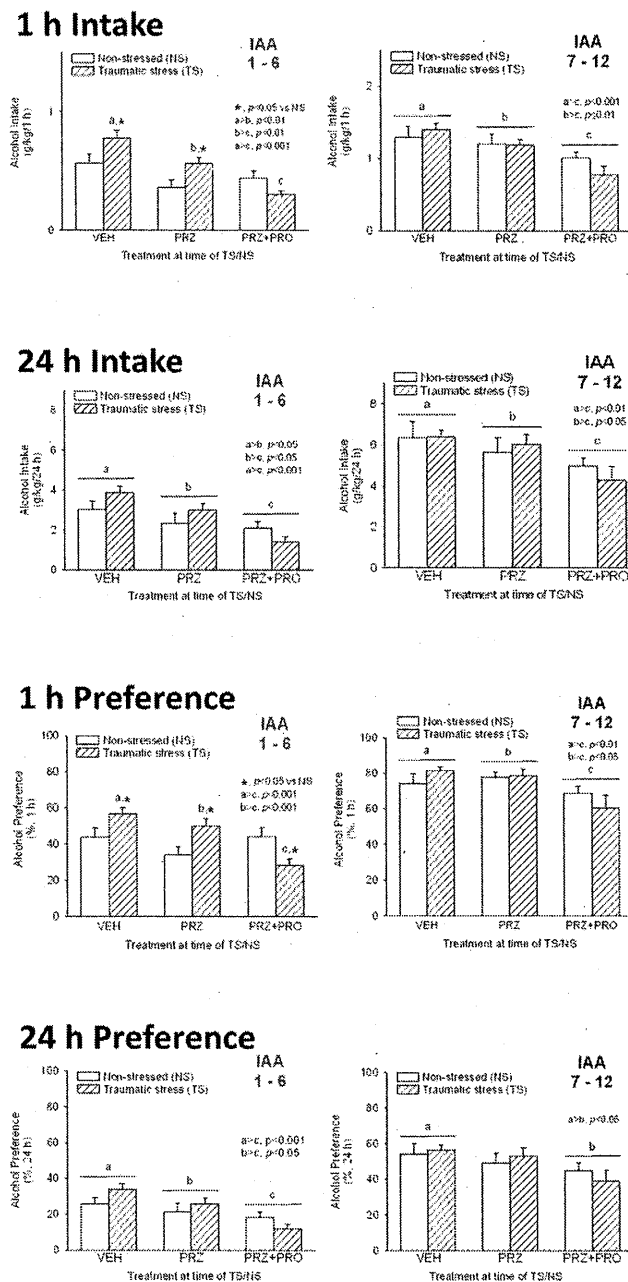


FIG. 10

suppressed by PRZ+PRO ($p < 0.01$ vs VEH, $p < 0.05$ vs PRZ) treatment at the time of the single TS/NS exposure, independent of TS vs NS exposure. Each bar represents data from 15-35 rats.

Fig. 10, Row 3: Alcohol preference during the first hour of each IAA, averaged across IAA trials 1-6 (LEFT PANEL) and 7-12 (RIGHT PANEL). In the first 6 IAA trials (LEFT PANEL), 1 h alcohol preference in rats that had received non-stress treatment 8 weeks prior to initiation of IAA were not significantly different among rats that had received VEH vs PRZ vs PRZ+PRO treatment at the time of the NS treatment. TS treatment 8 weeks prior to initiation of IAA increased 1 h alcohol preference ($p < 0.05$ vs NS) in rats that had been treated with VEH or PRZ at the time of the TS exposure; in contrast, TS at 8 weeks prior to IAA decreased alcohol preference ($p < 0.05$ vs NS) in rats that had received PRZ+PRO at the time of the TS exposure. The 1 h alcohol preference in rats that had received TS was suppressed by PRZ+PRO treatment at the time of TS ($p < 0.001$ vs VEH or PRZ). In IAA trials 7-12 (RIGHT PANEL), average 1 h alcohol preference in rats was suppressed by PRZ+PRO ($p < 0.01$ vs VEH or PRZ) treatment at the time of the single TS/NS exposure, independent of prior TS/NS exposure. Each bar represents data from 15-35 rats.

A preliminary experiment demonstrated that PRO (5 mg/kg) at the time of TS did not suppress either 1 h or 24 h alcohol preference during IAA initiated 8 weeks later. A separate experiment demonstrated that PRZ+PRO treatment at the time of TS did not alter 1 h intake of 1% sucrose in the week following completion of IAA (data not shown).

Fig. 10, Row 4: Alcohol preference during the entire 24 hours of each IAA, averaged across IAA trials 1-6 (LEFT PANEL) and 7-12 (RIGHT PANEL). Average 24 h alcohol intake during IAA trials 1-6 (LEFT PANEL) was suppressed by PRZ+PRO treatment ($p < 0.001$ vs VEH, $p < 0.05$ vs PRZ), independent of TS vs NS exposure. In IAA trials 7-12 (RIGHT PANEL), 24 h alcohol intake was suppressed by PRZ+PRO ($p < 0.05$ vs VEH) treatment at the time of the single TS/NS exposure, independent of TS/NS exposure. Each bar represents data from 15-35 rats.

These results suggest that a single traumatic stress followed by weekly contextual reminders of the stress can increase voluntary alcohol drinking by male Wistar rats ≥ 8 weeks after the traumatic stress.

Treatment with the $\alpha 1$ -adrenergic antagonist prazosin (PRZ) or – especially – combination treatment with both PRZ and the β -adrenergic antagonist propranolol (PRO) to reduce noradrenergic signaling at the time of the traumatic stress can decrease or prevent this later development of increased alcohol drinking, even though the drugs were administered only at the time of the traumatic stress (i.e., 30 minutes before and again 2 h after the stress) whereas the increased alcohol drinking was expressed ≥ 8 weeks later. These results suggest that PRZ

or PRZ+PRO (or other treatments that decrease noradrenergic signaling) at the time of traumatic stress could potentially provide prophylaxis for alcohol use disorders that commonly accompany development of PTSD. In contrast, a preliminary study demonstrated that PRO alone did not suppress the TS-induced IAA alcohol drinking.

The doses and times of PRZ or PRZ+PRO administration before traumatic stress in this study were the same as the doses and times that we previously demonstrated to acutely decrease alcohol drinking in rats without producing sedating or motor effects.

Trauma memory testing before the IAA was initiated, at 8 weeks after the single traumatic shock (TS), confirmed that the TS + reminders (R) rats remembered the single TS and that the context of the TS remained aversive (expressed as increased avoidance behavior and increased defecation during R, each $p < 0.001$ relative to NS, results not shown). These results suggest that PRZ treatment at the time of TS can decrease subsequent development of increased voluntary alcohol drinking 8-12 weeks after TS, that this response to PRZ is markedly enhanced by co-treatment with PRO, and that these responses are probably not mediated by decreasing the perceived aversiveness of the TS or by decreasing memory of the TS.

PRZ or [PRZ+PRO] administration at the time of TS potentially may provide effective preventive treatment for TS-induced development of alcohol abuse and – perhaps – PTSD or other correlates of PTSD. Further investigations are warranted to address effects of treatments at other time points relative to TS and R, resolution of responses by TS-sensitive vs -resilient subjects, and mechanisms to identify potentially clinically-effective interventions.

We also continue evaluating extensive acoustic startle and behavioral test results to address potential correlations and predictive validity of characterizations before and after the rat PTSD model in determining development of a PTSD-like condition and/or increased alcohol intake.

Laboratory assistants (and calendar month efforts) participating in these studies:

Personnel	Year 1	Year 2	Year 3	NCE 1	NCE 2
Kristen Baumann			5.34 CM	12 CM	11.26 CM
Jennifer Burns		0.3 CM	8.54 CM		
Carrie Kincaid	10.96 CM	7.97 CM			
Shelby Johanson	4.55 CM	12 CM	11.34 CM	12 CM	11.13 CM

- **CHANGES, PROBLEMS, DELAYS AND PLANS TO RESOLVE THEM**

After > 37 years at the University of California at San Diego (UCSD), University of Washington (UW), and the Dept of Veterans Affairs (VA), it has become necessary for me to retire as PI of laboratory-based research. This mandated that the laboratory focus in the last year must shift to completing the animal work for these current studies while facilitating my technicians' search for and transition to other positions. Their departures necessitated an early cessation of laboratory work, resulting in the return to DoD of approximately \$20,000 of the Direct + Indirect costs of this program. I have spent the remaining months closing down my laboratory. As of Jan 1, 2019, my appointment with the Department of Veterans Affairs (VA) will convert to Without Compensation (WOC) status. The local VA research administration has committed to continued provision of my office, and I now plan to focus on writing papers. I will maintain my appointment in the Department of Psychiatry at the University of Washington (unsalaryed).

There have been no significant changes in objectives and scope of this project.

As discussed in previous reports, a change from the original proposal was the incorporation of osmotic minipumps for long-term drug administrations. However, unanticipated problems with this method subsequently required that this method be abandoned and the experiment for Task 2 was re-done using the originally proposed method of repetitive oral administration and also, as discussed in the Year 3 report, administering the alpha-1 adrenergic receptor, doxazosin, rather than prazosin. As discussed in this report, this approach was successful, demonstrating that doxazosin treatment decreased the development of IAA alcohol drinking. However, suggestive evidence that this suppression of alcohol drinking by moderate dose doxazosin treatment was dependent upon pre-treatment acoustic startle response amplitude was not clear.

The order of the studies was changed in the Year 1 progress report and in the SOW revised at that time. At present the animal and laboratory work needed for all studies are complete.

As also previously noted in the Years 1 and 2 reports, it took longer than anticipated to recruit, hire (6 months after the award notice), process and train one new staff member (Shelby Johanson) in the first year, introducing delays at the start of the project. In Year 2, my long-time Laboratory Manager/Research Scientist, Carrie Kincaid, left my lab for a higher paying position as Research Manager for an extensive clinical research program at this VA medical center. After having her working with me for 10 years, this was of course highly disruptive. We then recruited, hired, processed and trained her replacement, Jennifer Burns, but she left the lab after 6 months when she received a fellowship to enter a neuroscience PhD program in Pittsburgh. Consequently, it was again

necessary to hire, process and train her replacement, Kristen Baumann. These repetitive personnel changes and the time that it has taken to get the new personnel up to speed and operating efficiently caused discontinuity, problems and delayed progress, so much so that required two 1-year no cost extensions. As discussed above, the time-intensive process of closing down my laboratory work and lab midway through this past year has further limited the function of my research program, pushing back much of the writing of papers until "retirement" in the new year.

▪ **SUMMARY DISCUSSION**

The first year was used for personnel recruitment and training, implementation of all necessary methodologies, completion of the first study (which is key to interpreting all subsequent studies), and initiating subsequent studies - each of which is an individually long-term study (ranging from months to greater than a half-year for each of multiple temporally-overlapping cohorts of subjects within each study). In the second year another large key study progressed well and initial results were enthusiastically received when presented at the annual meeting of the International Society of PsychoNeuroEndocrinology, a meeting that was focused on effects of stress on the brain. The final cohorts of animals have now completed all trials in this study and analyses of interactions of PTSD-like responses with alcohol drinking are underway. All results thus far have been reported initially in a series of presentations and published abstracts listed in Section 6. As discussed in the CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM section, we now shift to preparing manuscripts for publication.

4. KEY RESEARCH ACCOMPLISHMENTS

We discuss here the key research accomplishments in the final funding period + no-cost extension period. The previously reported accomplishments in years 1 and 2 are then included in the overall discussion presented in the following section: 5. Conclusion.

- The key new accomplishment was our demonstration that the effect of acute pharmacologic reduction of noradrenergic signaling on increased voluntary alcohol drinking is dependent on the individual rat's history of previous stresses as well as on the individual rat's previous history of repetitive high dose alcohol drinking and/or the associated development of compulsive alcohol drinking. This finding is important because it informs clinical trials in the path to implementing prazosin or other noradrenergic treatments to decrease alcohol abuse, since there has been large individual variability in responses to prazosin and doxazosin. The animal work for this part of the project has been completed. As we initially reported at the 2017 Annual Meeting of the Research Society on Alcoholism symposium organized by the PI, entitled "The Neglected

Catecholamine: Noradrenergic Mechanisms in Regulation of Alcohol-Related Behaviors”, rats that received alcohol in the IAA model for 24 weeks developed markedly elevated levels of alcohol drinking that was compulsive-like (i.e., it was resistant to adulteration of the alcohol with bitter tasting quinine) whereas rats that received continuous access to alcohol did not develop elevated levels of consumption and the consumption was not compulsive-like. Furthermore, the compulsive-like alcohol drinking of the IAA rats was suppressed by doxazosin treatment, whereas non-compulsive alcohol drinking in the rats receiving continuous alcohol access was actually increased by doxazosin treatment. These results were surprising, but consistent with results of a recent clinical study in the laboratory of Lorenzo Leggio, MD PhD (which were also presented in our symposium at the 2017 Research Society on Alcoholism meeting) - i.e., Dr Leggio demonstrated that alcoholic subjects who are family history (+) for alcoholism continue to drink high levels of alcohol even in the clinical trial setting (i.e., they exhibit evidence of compulsive drinking) and also respond to doxazosin with decreased alcohol drinking, whereas alcoholic subjects who are family history (-) exhibit markedly decreased alcohol drinking in the clinical trial setting (i.e, the clinical setting produces a large placebo effect consistent with non-compulsive drinking) but doxazosin paradoxically increases this alcohol drinking. The surprising results with our rat model thus are remarkably consistent with clinical evaluations with alcoholic subjects. Furthermore, our results in this study complemented results in our study demonstrating that although prazosin administration to rats that had received prior traumatic stress produced decreased alcohol drinking, prazosin administration to rats that had not received prior traumatic stress actually increased alcohol drinking. As highlighted by the Discussant/Question Moderator for the symposium, Dr. Raye Litten of the National Institute for Alcohol Abuse and Alcoholism (NIAAA), these results strongly suggest that a subset of subjects who are predisposed to develop compulsive alcohol drinking due to genetics and/or traumatic stress history may be the subset of otherwise difficult-to-treat subjects most appropriate for treatment to reduce noradrenergic signaling with prazosin or doxazosin.

- Another key accomplishment in the final period was based on completing a thorough analysis of experimental results demonstrating that pharmacologic reduction of noradrenergic signaling at the time of a single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress and long after the brief pharmacotherapy at the time of the trauma. This finding is important because it reveals a potential prophylactic approach in preventing effects of traumatic

stress on subsequent alcohol drinking. Perhaps even more important, it also reveals additional potential clinical interventions that now can be readily tested with the model that we have developed, such as prevention of PTSD responses by suppressing noradrenergic signaling at other time points (e.g., in the time period immediately following a traumatically stressful experience to block development of PTSD or sequelae, or at the time of reminders to facilitate extinction therapy). Analyses of this study are now complete and a post-doctoral fellow (Dr. Rebecca Hendrickson) is now preparing a corresponding manuscript.

- Additional new findings in this final period were key to the successful completion of other studies, even though they introduced delays. First, demonstration that repetitive oral administration of doxazosin - which has a longer half-life than prazosin (allowing daily administration in a piece of sweetened gelatin containing the doxazosin that the rats readily consumed) effectively decreases development of increased alcohol drinking. Second, administration of traumatic stress at 1 week after removal of liquid diet containing alcohol, a model of previous alcohol abuse effects on response to traumatic stress, was unexpectedly found to block subsequent development of a PTSD-like syndrome (compared to TS administered without prior chronic alcohol consumption), similar to the previous (original) study with TS administered only 5 hours after chronic consumption of alcohol.

5. CONCLUSION:

The key results reported for Year 1 were consistent with the hypothesis that is central to all other studies in this research project, i.e. that hyper-responsiveness characteristic of PTSD, alcohol withdrawal/abstinence, and increased noradrenergic activation contributes to development of increased alcohol drinking. These results provided the conceptual basis for a potential approach to prospectively identifying individuals – including individuals with PTSD - at increased risk for future alcohol use disorders, thus allowing development and implementation of potential preventive interventions.

The key result from a large experiment primarily conducted in Years 2 and 3 now provides evidence for a promising potential preventive intervention. We have previously demonstrated that a single episode of traumatic stress in our rat PTSD model can produce sustained marked increases in hyper-responsiveness reflected in acoustic startle response, which is noradrenergic activation dependent; this subsequent key result, i.e., that reduction of noradrenergic signaling at the time of single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress, demonstrates a potential pharmacologic intervention for preventing at least the subsequently increased alcohol drinking following a traumatic stress. With the addition

of a post-doctoral Fellow to our research group, we continue to evaluate what other aspects of PTSD-like rat behavior also respond to this pharmacologic intervention. The new key finding then also suggests subsequent related questions, such as “would treatment only immediately after a trauma also be effective?”, or “would treatment only at the time of each contextual reminder also be effective?”, and “what mechanisms are involved?” Addressing these subsequent questions would inform potentially effective pharmacotherapy in cases where traumatic stress had already recently occurred, or perhaps as an adjunct to subsequent PTSD psychotherapy. Together, these two initial key results also provide the conceptual bases for potential prospective identification of individuals – including individuals with PTSD – at increased risk for future alcohol use disorders, and then potentially applying preventive and – possibly - therapeutic pharmacologic intervention.

An additional key result in a study initiated in Year 3 revealed that the response to reduction of noradrenergic signaling depended on previous history of stress exposure. Rats that had been exposed to traumatic stress and subsequent contextual reminders in the rat PTSD model subsequently exhibited increased voluntary IAA alcohol drinking, and administration of prazosin or prazosin+propranolol acutely suppressed this drinking. However, rats that had not experienced traumatic stress did not exhibit such elevated IAA drinking and this voluntary alcohol drinking was not significantly suppressed by prazosin or prazosin+propranolol. We later repeated this important experiment, with the same results. Together, these results inform - and provide a pre-clinical model for further investigating – the interpretation of variable responses to prazosin that have been reported in clinical investigations.

This investigation of variable responses to suppression of noradrenergic signaling was advanced further in the no-cost extension with the demonstration that rats with a long history of intermittent high-dose alcohol drinking that led to compulsive alcohol drinking responded to acute doxazosin treatment by decreasing alcohol intake, whereas rats with the same duration of continuous access to alcohol conversely increased alcohol intake in response to doxazosin. This result is consistent with recent evidence that alcoholic patients with a dense family history of alcoholism, who also exhibit compulsive alcohol drinking (e.g., they maintain high alcohol intake even in a clinical trial context) decrease alcohol drinking in response to doxazosin, whereas alcoholic patients without a dense family history of alcoholism and without evidence of compulsive alcohol drinking (e.g., they drink little alcohol in the clinical trial setting) conversely increased alcohol intake in response to doxazosin. The PI co-organized and presented in a symposium at a recent Research Society on Alcoholism meeting, focused on this variability in responses to prazosin and prospective prediction/evaluation of who is most likely to respond. These studies are already having a significant impact on the

direction of research and clinical implementation related to prazosin and doxazosin treatments

As discussed above in the CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM section, manuscripts from the unpublished studies are in preparation.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Manuscripts

Lay press: Nothing to report

Peer-Reviewed Scientific Journals:

Rasmussen DD, Kincaid CC. Acoustic startle in alcohol-naïve male rats predicts subsequent voluntary alcohol intake and alcohol preference. *Alcohol Alcohol.* 50: 56-61, 2015 (doi: 10.1093/alcalc/agu065; PMID: 25305255)

Rasmussen DD, Kincaid CL, Froehlich JC 2017 Prazosin prevents increased anxiety behavior that occurs in response to stress during alcohol deprivations. *Alcohol Alcohol* 52:5-11. doi:10.1093/alcalc/ag082. Epub 2016 Oct 26. PMCID: 5169035. This paper was derived from work supported by another grant but it supports and complements the studies in this project and thus is included here.

Invited Articles: Nothing to report

Published abstracts:

Rasmussen DD, Johanson SS, Kincaid CL. Reduction of α 1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking. *Psychoneuroendocrinology* 61: 53, 2015 (doi: 10.1016/j.psyneuen.2015.07.535; PMID: 26383421)

Rasmussen DD, Johanson SS, Burns JL, Kincaid CL. Reduction of α 1- and β -adrenergic signaling at the time of traumatic stress reduces subsequent development of increased alcohol drinking in rats. *Alcohol.Clin.Exp.Res.* 40 (S1):198A, 2016. PMID not assigned.

Gonzales R, Rasmussen D (organizers). The neglected catecholamine: noradrenergic mechanisms in regulation of alcohol-related behaviors. *Alcohol.Clin.Exp.Res.* 41 (S1): 13A, 2017. PMID not assigned.

Rasmussen DD. Variability in alcohol drinking responses to suppression of noradrenergic signaling in outbred rats. *Alcohol.Clin.Exp.Res.* 41 (S1):236, 2017. PMID not assigned.

Rasmussen DD, SS Johanson, KK Baumann. A single brief traumatic stress produces long-term changes in noradrenergic

regulation of alcohol drinking in male Wistar rats.

Alcohol.Clin.Exp.Res. 42 (S1):91A, 2018. PMID not assigned.

Rasmussen DD, KK Bauman, SS Johanson. Qualitative effects of acute doxazosin treatment on alcohol drinking in Wistar rats is dependent upon long-term drinking pattern and/or level.

Alcohol.Clin.Exp.Res. 42 (S1):91A, 2018. PMID not assigned.

b. Presentations:

The PI and a PTSD clinical investigator colleague and consultant on this project, Dr. Murray Raskind, together presented a joint seminar in 2015 at the VA Puget Sound Health Care System (VAPSHCS), Mental Illness Research, Education and Clinical Center (MIRECC) and the VAPSHCS division of Research and Development in affiliation with the Seattle Institute of Biomedical and Clinical Research (SIBCR), entitled "Translation goes both ways; prazosin treatment from humans to rats and back". This presentation was not published.

The PI presented work described in an appended published abstract to the 2015 Annual Meeting of the International Society for PsychoNeuroEndocrinology: Rasmussen DD, Johanson SS, Kincaid CL. Reduction of α 1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking. Psychoneuroendocrinology 61: 53, 2015 (doi: 10.1016/j.psyneuen.2015.07.535; PMID: 26383421)

The PI presented work described in an appended published abstract to the 2016 Annual Meeting of the Research Society on Alcoholism: Rasmussen DD, Johanson SS, Burns JL, Kincaid CL. Reduction of α 1- and β -adrenergic signaling at the time of traumatic stress reduces subsequent development of increased alcohol drinking in rats. Alcohol.Clin.Exp.Res. 40 (S1):198A, 2016. PMID not assigned.

The PI was Co-Organizer (together with Reuben Gonzales PhD) of a symposium presented at the 2017 annual meeting of the Research Society on Alcoholism, an international society: The Neglected Catecholamine: Noradrenergic Mechanisms in Regulation of Alcohol-Related Behaviors. Alcohol.Clin.Exp.Res. 41 (S1): 13A, 2017. PMID not assigned.

The PI presented a lecture at the 2017 annual meeting of the Research Society on Alcoholism work, described in an appended published abstract: Rasmussen DD. Variability in alcohol drinking responses to suppression of noradrenergic signaling in outbred rats. Alcohol.Clin.Exp.Res. 41 (S1):236, 2017. PMID not assigned.

The PI presented work described in an appended published abstract to the 2018 Annual Meeting of the Research Society on Alcoholism: Rasmussen DD, SS Johanson, KK Baumann. A single brief traumatic stress produces long-term changes in noradrenergic regulation of alcohol drinking in male Wistar rats. Alcohol.Clin.Exp.Res. 42 (S1):91A, 2018. PMID not assigned.

The PI presented work described in an appended published abstract to the 2018 Annual Meeting of the Research Society on Alcoholism: Rasmussen DD, KK Bauman, SS Johanson. Qualitative effects of acute doxazosin treatment on alcohol drinking in Wistar rats is dependent upon long-term drinking pattern and/or level. Alcohol.Clin.Exp.Res. 42 (S1):91A, 2018. PMID not assigned.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report

- 8. REPORTABLE OUTCOMES:** Our initial key finding in Year 1, that acoustic startle in alcohol-naïve rats is highly predictive of subsequent voluntary IAA alcohol drinking and preference, extends and complements the results of one of our previous studies demonstrating that pre-stress acoustic startle predicts development of rat PTSD-like further increased acoustic startle and plasma corticosterone response following a traumatic stress. The key finding from preliminary findings initially reported in Years 2-3 demonstrating that reduction of noradrenergic signaling at the time of a single traumatic stress prevents subsequent development of increased alcohol drinking long after the traumatic stress, extends and complements our Year 1 key finding by revealing a potential new pharmacotherapeutic approach for preventing at least the subsequently increased alcohol drinking following a traumatic stress. Together these results suggest that increased acoustic startle and associated increased anxiety - both of which are increased by noradrenergic activation - reflect underlying mechanisms that increase vulnerability to both PTSD and alcohol abuse. Together with our and others' previous results demonstrating that prazosin can decrease both voluntary alcohol intake and PTSD symptoms, these results strongly suggest that prazosin can be effective for both conditions and that an α 1-adrenergic receptor-mediated mechanism is at least one component of the common underlying mechanism, and thus an especially appropriate target for both prophylactic and therapeutic interventions. However, another key finding in Year 3 further refined this interpretation by demonstrating that the voluntary alcohol drinking response to decreasing noradrenergic signaling appears to be dependent upon prior history of traumatic stress experience. This important result was confirmed in an additional study during no-cost extension. It was then also demonstrated that rats that had developed "compulsive-like" drinking following prolonged repetitive high-dose alcohol drinking in an intermittent access model also decreased this alcohol drinking in response to doxazosin, whereas rats that received continuous access to alcohol over the same time period did not develop high levels of voluntary alcohol drinking and their alcohol drinking was conversely increased in response to doxazosin. Although this complicates interpretations of the overall results, it is consistent with the evidence that all clinical subjects do not respond equally to prazosin or doxazosin treatment, and that alcohol-dependent patients with a high density family history of alcoholism,

exhibiting consistent drinking of high levels of alcohol (e.g., compulsive alcohol drinking), decrease their alcohol drinking in response to doxazosin; in contrast patients without high density family history of alcoholism voluntarily decrease their drinking in a clinical study setting and conversely exhibit increased alcohol drinking in response to doxazosin. Our studies provide an effective preclinical model for further resolving this variability in responses, prospectively identifying who is most likely to respond, and developing new treatments, or effective treatment combinations (such as prazosin or doxazosin administered together with naltrexone, which we demonstrated in studies - supported by a separate NIH grant – to suppress alcohol drinking in female alcohol-preferring (P) rats even when prazosin alone did not). The animal work for all studies are now complete, although there have been setbacks due to unexpected personnel turnovers, to methodologic issues that have made it necessary to now repeat some of the experiments (in some cases, several times), and now, by my impending retirement that led to loss of my technicians, precipitating closing my laboratory (see CHANGES, PROBLEMS, DELAYS AND ACTIONS TO RESOLVE THEM). Our further development of the rat PTSD model, employing a single traumatic stress together with weekly brief contextual reminders of the stress will – together with the further characterization of PTSD-like responses in these studies – also provide a well-characterized experimental model for other labs investigating PTSD and alcohol abuse, alone or together. In addition, a) the findings, results and techniques of these studies are directly applicable to other investigations of the effects of stress or the evaluation of mechanisms contributing to voluntary alcohol and other drug abuse, b) the results of this investigation will facilitate translating prazosin (and doxazosin) treatment to clinical implementation in the treatment of PTSD and alcohol abuse, alone or together, and c) our results will ultimately improve overall understanding and effective treatment of alcoholism and PTSD, two conditions with profound negative social and economic impact.

9. **OTHER ACHIEVEMENTS:** A great deal of basic and clinical PTSD and related traumatic brain injury (TBI) research is based at the VA Puget Sound Health Care System (VAPSHCS) Mental Illness Research, Education and Clinical Center (MIRECC), providing ample ongoing collaborative opportunities. The PI works closely with a PTSD clinical investigator colleague, Dr. Murray Raskind, as well as alcohol clinical investigators (Drs. Andrew Saxon and Tracy Simpson) and a TBI clinical investigator, Dr. Elaine Peskind, within the VAPSHCS and MIRECC to facilitate translation of basic science findings in the current investigation to clinical testing and future clinical implementation as discussed in Section 8.

10. REFERENCES

Rasmussen DD, Kincaid CC. Acoustic startle in alcohol-naïve male rats predicts subsequent voluntary alcohol intake and alcohol preference. *Alcohol Alcohol.* 50: 56-61, 2015 (doi: 10.1093/alcalc/agu065; PMID: 25305255)

Rasmussen DD, Johanson SS, Kincaid CL. Reduction of α 1-adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking. *Psychoneuroendocrinology* 61: 53, 2015 (doi: 10.1016/j.psyneuen.2015.07.535; PMID: 26383421)

Rasmussen DD, Johanson SS, Burns JL, Kincaid CL. Reduction of α 1- and β -adrenergic signaling at the time of traumatic stress reduces subsequent development of increased alcohol drinking in rats. *Alcohol.Clin.Exp.Res.* 40 (S1):198A, 2016. PMID not assigned.

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Gonzales R, Rasmussen D (organizers). The neglected catecholamine: noradrenergic mechanisms in regulation of alcohol-related behaviors. *Alcohol.Clin.Exp.Res.* 41 (S1): 13A, 2017. PMID not assigned.

Rasmussen DD. Variability in alcohol drinking responses to suppression of noradrenergic signaling in outbred rats. *Alcohol.Clin.Exp.Res.* 41 (S1):236, 2017. PMID not assigned.

Rasmussen DD, SS Johanson, KK Baumann. A single brief traumatic stress produces long-term changes in noradrenergic regulation of alcohol drinking in male Wistar rats. *Alcohol.Clin.Exp.Res.* 42 (S1):91A, 2018. PMID not assigned.

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11. **APPENDICES:** Six published abstracts and two published paper, listed above in Section 6, are appended.

Acoustic Startle in Alcohol-Naïve Male Rats Predicts Subsequent Voluntary Alcohol Intake and Alcohol Preference

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(Received 19 June 2014; in revised form 26 August 2014; accepted 29 August 2014)

Abstract — **Aims:** Acoustic startle response in rats is used to model sensorimotor reactivity. The aim of the study was to determine whether acoustic startle response in alcohol-naïve rats predicts subsequent increased voluntary alcohol drinking or alcohol preference. **Methods:** Startle responses to 90, 95 and 100 decibel (dB) white noise stimuli presented in counterbalanced semi-randomized order were tested in alcohol-naïve young adult male Wistar rats before voluntary alcohol intake was established with an intermittent alcohol access (IAA) model. **Results:** Startle amplitude in response to 95 or 100 dB stimuli was positively correlated with subsequent alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split) pre-IAA startle amplitude in response to 95 or 100 dB stimuli developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA, relative to rats with low pre-IAA startle amplitude. **Conclusion:** Startle response to moderate acoustic stimuli can be a predictive index of vulnerability to developing increased alcohol drinking.

INTRODUCTION

Begleiter and Porjesz (1999) proposed that sensorimotor hyper-reactivity is a key feature of the simplest model of the neuronal milieu underlying a predisposition to alcoholism. This hypothesis is consistent with evidence that sensorimotor hyper-reactivity expressed as enhanced acoustic startle response is characteristic of abstinent alcoholics (Krystal *et al.*, 1997) and is associated with family history of alcoholism (Pfefferbaum *et al.*, 1991; Grillon *et al.*, 1997). Rats selectively bred for alcohol preference and high voluntary alcohol drinking (McKinzie *et al.*, 2000; Chester *et al.*, 2004; Aciewicz *et al.*, 2012), and post-dependent rats experiencing either acute alcohol withdrawal or prolonged imposed alcohol abstinence (Rassnick *et al.*, 1992; Rasmussen *et al.*, 2005) also exhibit increased acoustic startle response.

Enhanced startle is associated with increased brain noradrenergic activation (Stevens *et al.*, 1994), and brain noradrenergic activation contributes to increased voluntary alcohol drinking (Walker *et al.*, 2008; Rasmussen *et al.*, 2009; Simpson *et al.*, 2009; Froehlich *et al.*, 2013; O'Neil *et al.*, 2013). Enhanced startle is also correlated with the increased anxiety (Morgan *et al.*, 1993; Davis *et al.*, 1997) that is common to many alcoholics (Cloninger, 1987; Kushner *et al.*, 2000) and that is a major risk factor for alcohol abuse (Koob and Le Moal, 1997). Furthermore, anxiety-related behavior in rats has been demonstrated to predict alcohol drinking under several schedules of alcohol access (Hayton *et al.*, 2012). We thus hypothesized that characterization of startle response may facilitate prospective identification of vulnerability to developing increased voluntary alcohol drinking and also may provide a basis for determining mechanisms mediating development of some alcohol use disorders. Accordingly, we investigated whether prospectively determined acoustic startle response in alcohol-naïve rats was correlated with subsequent increased voluntary alcohol drinking or increased alcohol preference in an intermittent alcohol access (IAA) model. IAA, in which rats have access to 2-bottle choice (water vs 20% alcohol) home cage alcohol drinking for three 24-h sessions/week, separated by at least 24 h (e.g. Monday, Wednesday, Friday),

has been reported to induce outbred Wistar rats to escalate alcohol intake over repetitive access sessions to achieve alcohol intake at individually variable high levels accompanied by high alcohol preference and blood alcohol concentrations (BACs) comparable to those achieved by selectively bred alcohol-preferring (P) rats, and has been suggested to effectively model some human alcohol use disorders (Wise, 1973; Simms *et al.*, 2008).

MATERIALS AND METHODS

Animals

Twenty-three alcohol-naïve young adult male Wistar rats (Simonsen Labs, Gilroy, CA, USA) weighing 285 ± 3 g were housed 2/cage in plastic shoebox cages with controlled temperature ($21 \pm 1^\circ\text{C}$) and a 12 h/12 h light/dark cycle (lights off at 0900 h). Standard rodent chow (Laboratory Rodent Diet #7001, Harlan Teklad, Madison, WI, USA) and water were available *ad libitum* throughout the study. All experimental procedures were approved by the Veterans Administration Puget Sound Health Care System Institutional Animal Care and Use Committee and conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Acoustic startle testing system

Acoustic startle was tested with an SR-LAB Acoustic Startle System (SDI, San Diego, CA, USA) using a slight modification of methods we previously reported (Rasmussen *et al.*, 2008). Each SR-LAB test chamber includes a ventilated sound-attenuated cabinet containing a clear plastic cylindrical rat enclosure mounted on a piezoelectric accelerometer that detects muscle twitch in response to a brief pulse of white (mixed frequency) noise produced by a tweeter inside the cabinet. The force exerted on the accelerometer is digitized and expressed in millivolt (mV) units. Each startle response signal generated by the accelerometer was recorded as 65 consecutive 1 ms recordings, starting at the onset of each 40-ms startle tone. Results were analyzed as maximum peak

amplitude. Startle stimulus and background white noise levels were calibrated with a Radio Shack Digital Sound Level Meter (33–2055; RadioShack Corp., Fort Worth, TX, USA) placed in the center of the cylindrical rat enclosure. Each of 8 SR-LAB test chambers was calibrated before each use to provide 450 mV response to a consistent test stimulus provided by an SDI Standardization Unit (SDI, San Diego, CA, USA). Each SR-LAB test chamber and rat enclosure was cleaned with 0.5% Liquinox (Alconox, Inc., New York, NY, USA) before each use.

Pre-test acclimation

After acclimation to the animal colony room and the reversed light/dark cycle for 3 weeks, rats were transferred to a dark testing room adjoining the colony room for 90 min at 1.5–3 h after lights-off on each of 5 days prior to testing, with ambient 60 decibel (dB) white noise produced by a White Noise Generator (SDI, San Diego, CA, USA). On the first 4 days, each rat was acclimated to a dark SR-LAB test chamber for 5 min with 60 dB white background noise (produced by the tweeter inside the chamber) before being returned to the colony room, housed with the same cage mate. On the fifth day, each of the rats was likewise transferred to the dark testing room with ambient 60 dB white noise, placed in the dark SR-LAB test chamber for 5 min with 60 dB white background noise, and then exposed to 10 presentations of 40 ms 95 dB white noise pulses presented at 30 s intervals before being returned to the colony room. The goal of this initial exposure to acoustic pulses was to minimize effects of novelty stress in the subsequent acoustic startle testing with similar white noise pulses. Immediately following completion of the acclimation process, each rat was returned to the same colony room but individually housed in a plastic shoebox cage. All procedures during the acclimation and subsequent acoustic startle testing and IAA were conducted under dim red illumination.

Acoustic startle testing

Startle testing was conducted 7–10 days after completion of the pre-test acclimation. On the test day, rats were again transferred to the dark testing room with 60 dB background white noise. After 90 min, each rat was placed in a dark SR-LAB testing chamber with 60 dB background white noise for 5 min before quantitation of startle responses to 10 presentations each of 40 ms 90, 95 or 100 dB white noise pulses at 30 s intervals, with one pulse of each intensity (i.e. 90, 95 or 100 dB) in

counterbalanced order within each of 10 sequential sets of three pulses (Table 1), with 60 dB background white noise between pulses. Thus, there were a total of 30 startle tests at 30 s intervals, with 10 tests/each of responses to 90, 95 or 100 dB pulses distributed in counterbalanced order over a 15-min period.

Alcohol drinking

Three weeks after startle testing, 2-bottle choice access to 20% (v/v) alcohol vs water was provided for 24 h/day, 3 days/week (M, W, F)—i.e. an IAA model (Wise, 1973; Simms *et al.*, 2008). The alcohol solution was prepared by diluting 95% alcohol (ethanol; Decon Labs, King of Prussia, PA, USA) with deionized water to make a 20% (v/v) solution. Alcohol (20%) and water were presented in ball-bearing sipper tubes, with positions of the tubes alternated in sequential alcohol access periods to control for potential side preferences. On days when alcohol was not provided, the rats had access to water only. On days when alcohol and water intakes were characterized for analysis, daily fluid intakes were determined by weighing each tube to the nearest 0.1 g. Alcohol and water tubes were also placed on two empty cages to determine loss due to spillage/leakage and evaporation; average losses in these two cages on each day were subtracted from intakes for that day. Net daily alcohol intake was converted to g alcohol/kg body weight. After 36 alcohol access days (i.e. 12 weeks, when stable alcohol intake was achieved) alcohol intake and alcohol preference (ml of alcohol intake/[ml of alcohol intake + ml of water intake]) were determined over the next 3 alcohol access days to characterize the relationships of each rat's alcohol intake and alcohol preference relative to its pre-IAA acoustic startle responses. One rat did not establish significant daily alcohol drinking (alcohol intake was <1 g/kg/day on all days evaluated) and was excluded from further analyses.

Data analyses

Startle amplitude in response to presentations of 90, 95 or 100 dB acoustic pulse intensities in counterbalanced order within each of 10 sequential sets of 3 pulse presentations were initially evaluated by two-way (set X pulse intensity) repeated measures analysis of variance (ANOVA) with repeated measures on sets (1–10) and pulse intensities (90, 95 or 100 dB). There was a significant effect of intensity, $F(2, 42) = 63.7$, $P < 0.001$, but no significant effect of set and no significant intensity $\times$ set interaction. Since startle amplitude in response to each of the acoustic stimulus intensities was independent of presentation time (set) within the 15 min test period, the average of all 10 responses to each stimulus intensity was used in subsequent analyses of the relationships between pre-IAA acoustic startle response vs IAA alcohol intake or alcohol preference. Similarly, IAA alcohol intake or alcohol preference on the 3 alcohol access days in IAA week 13 was analyzed by one-way ANOVA with repeated measures on day; there were no significant effects of day on either alcohol intake or alcohol preference, so 3-day average alcohol intake or 3-day average alcohol preference was likewise used in subsequent analyses of relationships between IAA week 13 alcohol intake or alcohol preference vs pre-IAA acoustic startle amplitude.

Pre-IAA startle amplitude in response to presentations of either 90, 95 or 100 dB stimuli was each compared with subsequent alcohol intake or alcohol preference by Pearson Product Moment Correlation Analysis. The pre-IAA startle response to

Table 1. Order of acoustic stimuli presentations

Sequential sets of 3 acoustic stimuli	Counterbalanced order of stimuli within sets (dB)		
1	90	95	100
2	95	100	90
3	100	90	95
4	90	95	100
5	95	100	90
6	100	90	95
7	90	95	100
8	95	100	90
9	100	90	95
10	90	95	100

There were 30 s intervals between stimuli within each set as well as between each sequential set.

90, 95 or 100 dB stimuli in rats grouped on the basis of high vs low (median split, $n = 11/\text{group}$) IAA week 13 alcohol intake was further compared by two-way (high vs low alcohol intake $\times$ stimulus intensity) ANOVA with repeated measures on stimulus intensity (90, 95, 100 dB). The pre-IAA startle amplitude in response to 90, 95 or 100 dB stimuli in rats grouped on the basis of high vs low (median split, $n = 11/\text{group}$) IAA week 13 alcohol preference was likewise compared by two-way (high vs low alcohol preference $\times$ stimulus intensity) ANOVA with repeated measures on stimulus intensity (90, 95, 100 dB). The IAA week 13 alcohol intake or alcohol preference of rats grouped on the basis of high vs low (median split, $n = 11/\text{group}$) pre-IAA startle amplitude in response to either 90, 95 or 100 dB stimuli was each analyzed by Student *t*-test (median splits of startle responses to each of the three stimulus intensities did not in each case identify the same animals to be included in the high vs low startle response groups, so two-way ANOVA testing could not be performed); Bonferroni corrections were not applied to individual *t*-tests.

All analyses were conducted using Sigmaplot Version 11 software (Systat Software, Inc., Chicago, IL, USA) with significance accepted at $P < 0.05$. Data are presented as mean $\pm$ SEM.

RESULTS

IAA alcohol intake and alcohol preference

Initial (i.e. IAA week 1) average (M, W, F) alcohol intake was 0.95 ± 0.16 g/kg/24 h and alcohol preference was 0.07 ± 0.01 . By IAA week 13, alcohol intake had increased to 3.80 ± 0.32 g/kg/24 h ($P < 0.001$) and alcohol preference had increased to 0.40 ± 0.03 ($P < 0.001$).

IAA alcohol intake relative to pre-IAA startle response

IAA week 1 alcohol intake was not significantly correlated with pre-IAA startle amplitude elicited in response to 90 ($P = 0.18$), 95 ($P = 0.11$) or 100 ($P = 0.15$) dB stimuli. IAA week 13 alcohol intake relative to pre-IAA startle amplitude elicited in response to presentations of either 90, 95 or 100 dB stimuli is presented in the upper, middle or lower row, respectively, of Fig. 1.

Pre-IAA startle amplitude in response to 90 dB stimuli was modest and inconsistent; startle amplitude was not significantly correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, upper row, left). Grouping the rats on the basis of high vs low (median split) IAA week 13 alcohol intake revealed no alcohol intake-dependent significant difference in pre-IAA startle in response to 90 dB stimuli (Fig. 1, upper row, center; in the two-way ANOVA with repeated measures on stimulus intensity, there was a significant overall [alcohol intake (high, low) $\times$ stimulus intensity (90, 95, 100 dB)] interaction, $F[2, 40] = 5.1$, $P \leq 0.01$, but pre-IAA startle amplitude in response to the 90 dB stimulus was not significantly different between the high and low alcohol intake groups). Grouping on the basis of high vs low (median split) pre-IAA startle response to 90 dB stimuli likewise revealed no pre-IAA startle amplitude-dependent significant difference in IAA week 13 alcohol intake (Fig. 1, upper row, right).

Pre-IAA startle amplitude in response to 95 dB stimuli was positively correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, middle row, left; $P < 0.01$, $r =$

0.62). Rats with high (median split) alcohol intake in IAA week 13 had previously exhibited greater pre-IAA startle response to 95 dB stimuli, relative to rats with low alcohol intake in IAA week 13 (Fig. 1, middle row, center; $P < 0.001$). Consistent with this result, rats with high (median split) pre-IAA startle amplitude in response to 95 dB stimuli subsequently developed increased alcohol intake in IAA week 13, relative to rats with low pre-IAA startle response to 95 dB stimuli (Fig. 1, middle row, right; $P \leq 0.001$).

Pre-IAA startle amplitude in response to 100 dB stimuli also was positively correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, lower row, left; $P < 0.01$, $r = 0.55$). Rats with high (median split) alcohol intake in IAA week 13 had previously exhibited greater pre-IAA startle response to 100 dB stimuli (Fig. 1, lower row, center; $P < 0.001$). Consistent with this results, rats with high (median split) pre-IAA startle response to 100 dB stimuli subsequently developed increased alcohol intake in IAA week 13, relative to rats with low pre-IAA startle response to 100 dB stimuli (Fig. 1, lower row, right; $P < 0.01$).

IAA alcohol preference relative to pre-IAA startle response

IAA week 1 alcohol preference was not significantly correlated with pre-IAA startle amplitude elicited in response to 90 ($P = 0.19$), 95 ($P = 0.14$) or 100 ($P = 0.20$) dB stimuli.

IAA week 13 alcohol intake and alcohol preference were highly positively correlated, $r = 0.92$, $P < 0.001$. Further analyses of IAA week 13 alcohol preference relationships to pre-IAA startle responses were conducted identically to those in the preceding analysis of alcohol intake relationships to pre-IAA startle responses. Consistent with the high positive correlation between alcohol intake and alcohol preference, the results of analyses of alcohol preference vs pre-IAA startle responses, as detailed below, were essentially identical to the results of the preceding analyses of alcohol intake vs pre-IAA startle responses. Pre-IAA startle amplitude in response to 90 dB stimuli was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.05$, $r = 0.47$). Grouping the rats on the basis of high vs low (median split) IAA week 13 alcohol preference revealed no alcohol preference-dependent significant difference in pre-IAA startle response to 90 dB stimuli (in the two-way ANOVA with repeated measures on stimulus intensity, there was a significant overall [alcohol intake (high, low) $\times$ stimulus intensity (90, 95, 100 dB)] interaction, $F[2, 40] = 6.41$, $P < 0.01$, but pre-IAA startle in response to the 90 dB stimulus was not significantly different between the high and low alcohol intake groups). Grouping on the basis of high vs low (median split) pre-IAA startle response to 90 dB stimuli likewise revealed no pre-IAA startle amplitude-dependent significant difference in IAA week 13 alcohol preference.

Pre-IAA startle amplitude in response to 95 dB pulses was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.01$, $r = 0.57$). Rats with high (median split) alcohol preference in IAA week 13 had previously exhibited greater pre-IAA startle response to 95 dB stimuli, relative to rats with low alcohol preference in IAA week 13 ($P < 0.05$). Consistent with this result, rats with high (median split) pre-IAA startle response to 95 dB stimuli subsequently developed increased alcohol preference in IAA week 13, relative to rats with low pre-IAA low startle response to 95 dB stimuli ($P < 0.01$).

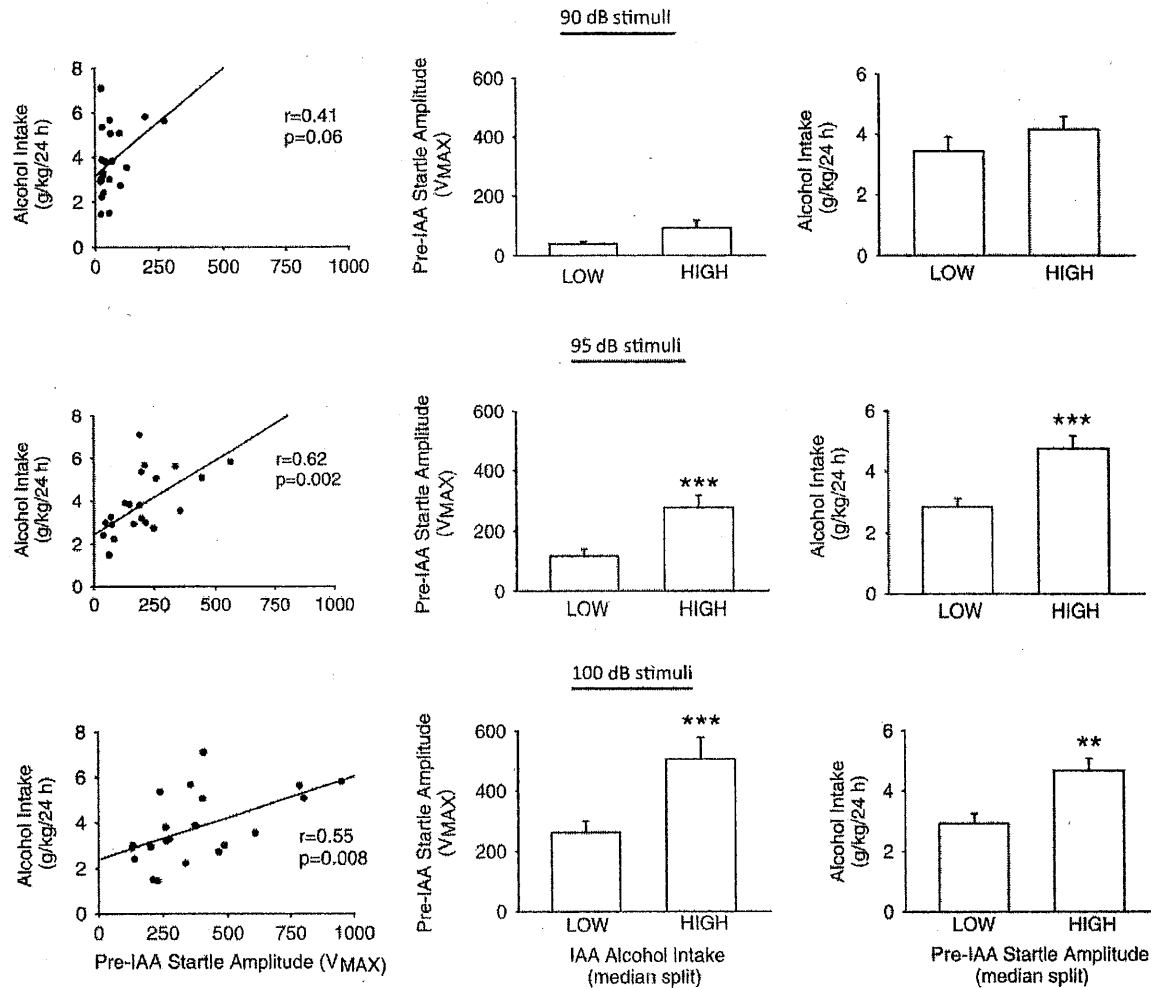


Fig. 1. Pre-IAA acoustic startle response vs alcohol intake following 3 months of IAA. Rows: The upper, middle and lower rows present analyses of pre-IAA responses to 90, 95 or 100 dB acoustic startle stimuli, respectively. Columns: The left panel in each row presents the correlation between pre-IAA acoustic startle amplitude vs IAA week 13 alcohol intake for all 22 rats. The center panel in each row presents the pre-IAA acoustic startle amplitude of rats grouped on the basis of low vs high (median split, $n=11$ rats/group) alcohol intake in IAA week 13. The right panel in each row presents the IAA week 13 alcohol intake of rats grouped on the basis of low vs high (median split, $n=11$ rats/group) pre-IAA acoustic startle amplitude. ** $P \leq 0.01$ vs Low, *** $P \leq 0.001$ vs Low.

Pre-IAA startle response to 100 dB stimuli also was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.01$, $r = 0.53$). Rats with high (median split) alcohol preference in IAA week 13 had previously exhibited greater pre-IAA startle response to 100 dB stimuli, relative to rats with low alcohol preference in IAA week 13 ($P < 0.001$). Consistent with this result, rats with high (median split) pre-IAA startle response to 100 dB stimuli subsequently developed increased alcohol preference in IAA week 13, relative to rats with low pre-IAA startle response to 100 dB stimuli ($P < 0.01$).

IAA alcohol intake relative to pre-IAA startle response to the first presentation of each stimulus intensity

Some previous investigations of alcohol drinking in rats have compared alcohol intake or preference relative to startle

amplitude in response to only the first presentation of an acoustic stimulus. In the current study, IAA week 13 alcohol intake or alcohol preference was not significantly correlated with pre-IAA startle in response to the first presentation of either 90 or 100 dB stimuli. However, pre-IAA startle amplitude in response to the first 95 dB stimulus was correlated with IAA week 13 alcohol intake ($r = 0.66$; $P \leq 0.001$) as well as alcohol preference ($r = 0.56$; $P < 0.01$).

DISCUSSION

In alcohol-naïve young adult male Wistar rats, acoustic startle amplitude in response to 40 ms pulses of white noise at intensities of 95 or 100 dB was positively correlated with subsequent voluntary alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split)

alcohol intake or alcohol preference following the 3 months of IAA had previously exhibited greater pre-IAA startle response to 95 as well as 100 dB stimuli, relative to rats with low alcohol intake or low alcohol preference. Conversely, rats with high (median split) pre-IAA startle response to 95 or 100 dB stimuli subsequently developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA.

Stimulus intensities in this investigation were based on the results of preliminary trials with young male Wistar rats in which 90 dB stimuli produced inconsistent small startle responses, 95 or 100 dB stimuli reliably produced relatively consistent sub-maximal startle, and a higher intensity stimulus (120 dB) produced maximal responses. The moderate 90, 95 and 100 dB stimuli were selected in order to avoid ceiling effects that could compromise ability to differentiate responses between animals, as suggested by a report that human startle amplitudes elicited by 90 dB, but not 114 dB, stimuli were positively correlated with number of previous alcohol detoxifications (Krystal *et al.*, 1997). It previously has been reported that male Wistar rats exhibited an inverted U-shaped curvilinear relationship between the startle response to an initial 120 dB acoustic stimulus vs later alcohol intake, and that startle habituation appeared to have predictive value regarding alcohol intake (Sandbak *et al.*, 2000). In the current study, habituation to repeated stimulus exposures was not apparent, and there were significant positive linear correlations between pre-IAA acoustic startle responses to 95 or 100 dB stimuli vs alcohol intake and alcohol preference following IAA. The apparent disparities between the Sandbak *et al.* (2000) study and the current study may be due to the differing stimulus intensities as well as to the incorporation of an initial session with exposure to repetitive moderate (95 dB) stimuli in advance of the testing trial in the current study in order to minimize novelty of the stimulus (consistent with clinical studies, in which the subjects are aware that they will hear acoustic stimuli during the testing trial). In addition, stimuli of three different intensities were presented in semi-random counterbalanced order throughout the 15 min trial in the current study, rather than consistent repetition of a single stimulus. It is also notable that the current study used an IAA model of alcohol drinking in which a relatively high concentration of alcohol (20%, v/v) was available on 3 intermittent days each week, considered to be a model for excessive alcohol drinking (Wise, 1973; Simms *et al.*, 2008).

Alcohol-naïve rats from lines selectively bred to prefer alcohol exhibit increased acoustic startle relative to selectively bred alcohol non-preferring rats (McKinzie *et al.*, 2000; Chester *et al.*, 2004; Aciewicz *et al.*, 2012). Sons of alcoholics likewise exhibited increased acoustic startle compared with sons of non-alcoholic parents (Grillon *et al.*, 1997). The current results suggest that mechanisms contributing to acoustic startle response have a functional role in the vulnerability to increased voluntary alcohol drinking, and that acoustic startle characterization can provide an index of sensorimotor hyper-reactivity and associated mechanisms that contribute to this increased alcohol drinking. Although these mechanisms remain to be resolved, it has been demonstrated that brain noradrenergic activation increases acoustic startle response (Stevens *et al.*, 1994) and also produces sensorimotor hyper-reactivity and anxiety (Redmond and Huang, 1979; Sullivan *et al.*, 1999) which are major risk factors for development of

alcohol use disorders (Cloninger, 1987; Koob and Le Moal, 1997; Begleiter and Porjesz, 1999; Kushner *et al.*, 2000). Conversely, suppression of noradrenergic signaling not only decreases acoustic startle responses (Gresack and Risbrough, 2011; Olson *et al.*, 2011) but also decreases alcohol drinking in rats and humans (Walker *et al.*, 2008; Rasmussen *et al.*, 2009; Simpson *et al.*, 2009; Froehlich *et al.*, 2013; O'Neil *et al.*, 2013) and blocks the expression of increased alcohol drinking in rats selectively bred for alcohol intake (Froehlich *et al.*, 2013). The consistent association of changes in acoustic startle, anxiety and increased alcohol drinking with changes in noradrenergic signaling suggests that noradrenergic activation may have a key role in mediating the correlation between acoustic startle amplitude and subsequent development of increased voluntary alcohol drinking.

The current results demonstrate that acoustic startle amplitude in response to moderately supra-threshold startle stimulus intensities administered to alcohol-naïve male Wistar rats is an effective predictive index for subsequent increased voluntary alcohol intake and alcohol preference in the IAA model. Acoustic startle response may be an especially useful index of the vulnerability to developing increased alcohol drinking because it is not dependent upon, and potentially confounded by, interactions with other behaviors. Importantly, acoustic startle is also well-characterized for use in humans (Krystal *et al.*, 1997; Grillon and Baas, 2003; Grillon *et al.*, 1998, 2005), providing translational utility.

These results may provide a useful model for investigating neurobiological mechanisms mediating initiation and development of excessive alcohol drinking, as well as provide the conceptual basis for a potential approach to prospectively identifying individuals at increased risk for future alcohol use disorders, thus allowing potential preventive intervention.

Funding — This work was supported in part by resources from the VA Puget Sound Health Care System, Seattle, Washington; US Army Medical Research Acquisition Activity CDMRP (contract number W81XWH-13-1-0126); and National Institutes of Health (grant number AA10567).

Conflict of interest statement. None declared.

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This paper was derived from work supported by another grant but it supports and complements the studies in this project and thus is included here.

Alcohol and Alcoholism, 2017, 52(1) 5–11

doi: 10.1093/alcalc/agw082

Advance Access Publication Date: 26 October 2016

Article



Article

Prazosin Prevents Increased Anxiety Behavior That Occurs in Response to Stress During Alcohol Deprivations

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Received 11 August 2016; Revised 6 October 2016; Editorial Decision 6 October 2016; Accepted 14 October 2016

Abstract

Aims: Stress-induced anxiety is a risk factor for relapse to alcohol drinking. The aim of this study was to test the hypothesis that the central nervous system (CNS)-active α_1 -adrenergic receptor antagonist, prazosin, would block the stress-induced increase in anxiety that occurs during alcohol deprivations.

Methods: Selectively bred male alcohol-preferring (P) rats were given three cycles of 5 days of *ad libitum* voluntary alcohol drinking interrupted by 2 days of alcohol deprivation, with or without 1 h of restraint stress 4 h after the start of each of the first two alcohol deprivation cycles. Prazosin (1.0 or 1.5 mg/kg, IP) or vehicle was administered before each restraint stress. Anxiety-like behavior during alcohol deprivation following the third 5-day cycle of alcohol drinking (7 days after the most recent restraint stress $\pm$ prazosin treatment) was measured by performance in an elevated plus-maze and in social approach/avoidance testing.

Results: Rats that received constant alcohol access, or alcohol access and deprivations without stress or prazosin treatments in the first two alcohol deprivations did not exhibit augmented anxiety-like behavior during the third deprivation. In contrast, rats that had been stressed during the first two alcohol deprivations exhibited increased anxiety-like behavior (compared with control rats) in both anxiety tests during the third deprivation. Prazosin given before stresses in the first two cycles of alcohol withdrawal prevented increased anxiety-like behavior during the third alcohol deprivation.

Conclusion: Prazosin treatment before stresses experienced during alcohol deprivations may prevent the increased anxiety during subsequent deprivation/abstinence that is a risk factor for relapse to alcohol drinking.

Short summary: Administration of prazosin before stresses during repetitive alcohol deprivations in male alcohol-preferring (P) rats prevents increased anxiety during a subsequent deprivation without further prazosin treatment. Prazosin treatment during repeated alcohol deprivations may prevent the increased anxiety that is a risk factor for relapse to alcohol drinking.

INTRODUCTION

Alcoholism co-occurs at high rates with anxiety disorders (Kushner *et al.*, 2000), anxious individuals report that they use alcohol primarily to control anxiety (Kushner *et al.*, 2000), and the tendency to develop alcohol dependence exhibits co-morbidity with increased anxiety and with central nervous system (CNS) disinhibition/hyperexcitability (Merikangas *et al.*, 1998; Sinha *et al.*, 1998; Begleiter and Porjesz, 1999). Increased anxiety is a major risk factor for alcohol relapse (Koob and LeMoal, 1997). Individuals with alcoholism commonly exhibit increased anxiety during abstinence (Koob and LeMoal, 1997; Kushner *et al.*, 2000) and report that relief of anxiety is a major reason for returning to alcohol drinking (Edwards *et al.*, 1972). Like humans, rats exhibiting increased anxiety-like behavior consume more alcohol than do rats exhibiting low anxiety behavior (Spanagel *et al.*, 1995) and rats selectively bred for high alcohol preference (e.g. P rats) exhibit higher levels of anxiety-like behavior than do alcohol non-preferring (NP) rats (Stewart *et al.*, 1993). This evidence strongly suggests that anxiety can contribute to increased alcohol drinking and relapse to alcohol drinking (but see Langen and Fink, 2004, for evidence that anxiety is not a reliable predictor of alcohol preference in all rat strains and experimental models).

Repeated episodes of alcohol drinking and withdrawal increase abstinence-induced symptoms in subjects with alcoholism (Ballenger and Post, 1978). An increase in abstinence-induced symptoms is also evident in rats, in which repeated alcohol deprivations have been proposed to sensitize or 'kindle' subsequent deprivation-induced anxiety-like behavior (for review, see Breese *et al.*, 2005a). Acute stress administered to rats during repetitive alcohol deprivations further increases this deprivation induction of anxiety behavior (Breese *et al.*, 2005a), leading to the suggestion that stress during alcohol withdrawal and abstinence can produce a high anxiety state that persists across repetitive alcohol withdrawal cycles and may contribute to relapse to alcohol drinking and alcohol dependence (Breese *et al.*, 2005a).

It has been reported that administration of corticotropin-releasing factor type 1 receptor (CRF₁) antagonists at the time of stress application blocks the stress-induced increases in anxiety behavior during subsequent alcohol deprivation (Overstreet *et al.*, 2005). Prazosin, an α_1 -adrenergic receptor antagonist, decreases brain CRF signaling (Itoi *et al.*, 1994) and is anxiolytic (Skelly and Weiner, 2014). We hypothesized that prazosin—a well-characterized, safe, well-tolerated and FDA-approved drug may be useful in reducing responses to stress during repetitive alcohol deprivations that lead to increased anxiety.

We previously demonstrated that prazosin treatment acutely and chronically decreases voluntary alcohol drinking in P rats (Rasmussen *et al.*, 2009; Froehlich *et al.*, 2013), alcohol drinking in a rat model of relapse to alcohol drinking (Froehlich *et al.*, 2015), alcohol seeking and drinking in operant paradigms (Verplaetse *et al.*, 2011), and drinking during acute withdrawal in alcohol-dependent outbred rats (Walker *et al.*, 2008). Prazosin treatment also facilitates abstinence in treatment-seeking alcohol-dependent men, as demonstrated in a study in which the subjects were unaware of their treatment condition (prazosin vs placebo) and there were no differences in side effects reported (Simpson *et al.*, 2009). In addition, prazosin decreases stress- and cue-induced alcohol craving in alcohol-dependent men and women (Fox *et al.*, 2012). The present study investigated whether prazosin, administered only at the time of stress during alcohol deprivation, would block increases in

anxiety behavior after alcohol reinstatement and an additional deprivation. Such a finding would suggest that treatment with prazosin could decrease alcohol drinking not only when administered at the time of alcohol intake, as previously demonstrated, but also potentially by suppressing stress-induced responses that facilitate subsequent alcohol deprivation-induced anxiety.

MATERIALS AND METHODS

Animals

Alcohol-naïve male P rats ($n = 40$) from generation 71 of selective breeding for alcohol preference were provided by the Alcohol Research Resource Center of the Indiana Alcohol Research Center. The rats were received at 30 days of age and were group housed (three/cage in clear plastic cages) in an isolated room with controlled temperature ($21 \pm 1^\circ\text{C}$) and reversed 12-h light/dark cycle (lights off at 10:00 h; procedures during dark periods were performed under dim red illumination). Standard rodent chow (Laboratory Rodent Diet 5001; PMI Nutrition International, Brentwood, MO) was available *ad libitum* at all times; water was available *ad libitum* at all times except as noted in the Alcohol Treatment section. Starting at 60–70 days, the rats were individually housed in clear plastic cages. Body weight (BW, 372 ± 8 g at start of study) was determined weekly. All experiments were approved by the VA Puget Sound Health Care System IACUC and conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Alcohol treatment

Alcohol solutions were prepared by diluting 95% alcohol (ethanol) with deionized water. Alcohol and water were presented in two glass drinking tubes (100 ml; BioServ, Frenchtown, NJ) with open-well side-arms extending through adjacent holes on the front of the cage. The rats initially received 3 days access to 10% (v/v) alcohol as the only source of fluid (i.e. alcohol in both drinking tubes), except for a Control group that never received alcohol. During subsequent two-bottle choice between water and 10% alcohol, positions of water and alcohol drinking tubes were alternated daily. Intake of alcohol or water was determined by weighing the drinking tubes to the nearest 0.1 g, with alcohol and water tubes on two empty cages providing estimated losses due to spillage and/or evaporation, which were subtracted from intakes. Net alcohol intake was converted to g alcohol/kg BW.

Experimental design

We modified a model originally used by Overstreet *et al.* (2005) to test drug effects on stress-induced facilitation of anxiety-like behavior during subsequent alcohol deprivation in P rats. As illustrated in Fig. 1 and reviewed in detail by Breese *et al.* (2005a), this model is based on a protocol in which three cycles of 5 days of voluntary

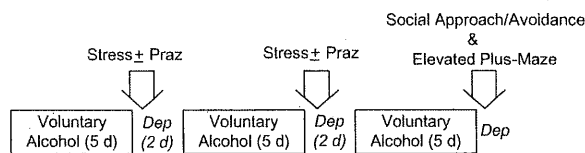


Fig. 1. Experimental protocol. Praz, prazosin; Dep, alcohol deprivation; d, days.

alcohol drinking are each followed by 2 days of alcohol deprivation, with or without restraint stress applied during the first and second alcohol deprivations; anxiety-like behavior is evaluated during a third alcohol deprivation. Drugs are administered before restraint stress during the first and second alcohol deprivations, but no drugs are administered in the third cycle of deprivation when anxiety-like behavior is tested. Consequently, anxiety-like behavior is tested 7 days after the most recent drug treatment.

Adult male P rats were divided into six treatment groups ($n = 6$ –7/group), counterbalanced on the basis of BW. Each group received access to two drinking tubes daily. The Control group received water in both drinking tubes throughout the study; a Constant Access group was provided with a continuous choice between water and 10% (v/v) alcohol in the two drinking tubes (this group served as a control for length of exposure to alcohol); and a Deprivations group received three cycles of 5 days of choice between water and 10% alcohol, interrupted by 2-day alcohol deprivations (i.e. water in both tubes during alcohol deprivation). The remaining three treatment groups received three cycles of 5 days of access to water *vs* 10% alcohol, interrupted by 2-day alcohol deprivations after cycles 1 and 2, with the addition of 1 h restraint stress starting 4 h after initiation of alcohol deprivations 1 and 2. These rats received 0 (vehicle alone), 1.0 or 1.5 mg/kg BW prazosin administered IP 30 min before restraint stress in cycles 1 and 2 as follows: Deprivation + Stress + Vehicle, Deprivation + Stress + 1.0 mg/kg Prazosin, or Deprivation + Stress + 1.5 mg/kg Prazosin. After the third cycle of 5 days of choice between water and 10% alcohol, a final alcohol deprivation was introduced and each rat was tested 4–5 h later for social approach/avoidance behavior, immediately followed by testing in an elevated plus-maze. All rats were acclimated to the testing room for 3 h with ambient 60 db white noise before any testing began.

Drug treatments

Prazosin HCl (Sigma-Aldrich Corp., St. Louis, MO) was dissolved in water and then diluted 1:1 with 90 mM lactate buffer (pH 5.2) before IP administration in a volume of 4 ml/kg at 30 min prior to restraint stress. Vehicle was 45 mM lactate buffer (pH 5.2). The IP doses of prazosin in this study were the same as the IP doses of prazosin that we have previously demonstrated to decrease alcohol drinking without producing sedation, motor effects, or malaise in male P rats (Rasmussen *et al.*, 2009, 2011; Froehlich *et al.*, 2015).

Restraint stress

Each rat was restrained for 1 h in a flexible clear plastic cone (Decapicone; Braintree Scientific, Inc., Braintree, MA) with the small open end of the cone positioned by the rat's nose and the wide end of the cone closed with two binder clips so the rat could not turn around or escape. Each restrained rat was placed in a clean cage in a dark room (with dim red illumination) separate from the colony room. Rats that did not receive restraint stress remained in their home cages.

The Social Approach/Avoidance Test

The Social Approach/Avoidance Test has been well characterized and extensively used as a test of rat anxiety-like behavior (Toth and Neumann, 2013). We conducted a three-chambered social approach test (Toth and Neumann, 2013) using a rectangular three-chambered arena (40 × 110 cm overall, with 30 cm high opaque

plastic walls); divided into a 40 × 20 cm central chamber separating two 40 × 45 cm end chambers by two clear plastic walls with guillotine doors. At the start of the trial, the arena is empty and the test rat is placed in the center chamber with both doors open so the rat can explore all three chambers for 5 min. The doors are then closed with the test rat confined in the center chamber while a 15 × 15 × 15 cm aluminum mesh cage, enclosing an unfamiliar male Wistar 'target' rat of the same age, is placed along the distal wall of one end chamber and an identical, but empty, mesh cage is placed along the distal wall of the other end chamber. The mesh cage allows visual, olfactory, auditory and some tactile contact with the unfamiliar target rat. A weighted canister is placed on top of each cage so the target rat cannot move the cage and the test rat cannot climb on top of either cage. The guillotine doors are then opened and the test rat is allowed to explore all three chambers for 5 min, choosing between spending time with the caged target rat, with the empty cage, or in the empty central chamber. The trial is conducted in dim (7–8 lux) light and is video-recorded with an overhead DynaView SSC-590 surveillance camera (Sony USA, New York, NY). Time that the test rat spends in close investigation of the target rat (nose within 2 cm of the cage containing the target rat, oriented toward and sniffing and/or actively examining the cage and enclosed rat) is considered social approach or interaction; time in similar close proximity to the empty cage in the opposite end chamber represents non-social investigation of a novel item.

Elevated plus-maze

This well-characterized and widely used test of rat anxiety-like behavior was conducted as we have previously reported (Rasmussen *et al.*, 2001). The Kinder Scientific (Poway, CA) elevated plus-maze has two closed arms (10 cm wide × 50 cm long) intersected by two perpendicular open arms (10 cm wide × 50 cm long), with a 10 × 10 cm central area. The closed arms have opaque black walls 50 cm high; the open arms were modified to include 1 cm clear plastic edges so the rat can easily turn around without slipping off the arm. This plus-shaped maze is elevated 80 cm above the floor on a center pedestal. The test is conducted in dim (7–8 lux) light immediately after the social interaction test (in the same room but separated by a curtain) and video-recorded with an overhead DynaView SSC-DC590 surveillance camera. The test is initiated by placing the rat in the center of the elevated plus-maze with its head directed toward a closed arm; decreased time on open arms and decreased entries to open arms during the 5-min test are considered to reflect increased anxiety-like behavior. Total arm entries into either open or closed arms reflect overall activity. Entrance to an open or closed arm is determined by all four paws leaving the central area.

Statistics

This study was designed to test the hypothesis that administration of prazosin would block the stress-induced increase in anxiety that occurs during alcohol deprivation, so all possible combinations of alcohol access, stress and prazosin treatments were not tested—i.e. the design is not fully factorial. Results of each test of anxiety-like behavior were compared among the six treatment groups by one-way analysis of variance (ANOVA) followed—when a significant overall *F* ratio was obtained—by Student–Newman–Keuls (SNK) pairwise comparisons. Analyses were performed with Sigmaplot 11 software (Systat Software, Inc., Chicago, IL) with significance accepted at $P \leq 0.05$. Data are presented as mean ± SEM.

RESULTS

Daily alcohol intake during days 11–15 of continuous alcohol access (Constant Access group) averaged 5.8 ± 0.5 g/kg BW/24 h. Alcohol intake during the third 5-day period of alcohol access after the 2nd alcohol deprivation (alcohol access days 11–15) averaged 7.1 ± 0.3 , 7.9 ± 0.3 , 7.5 ± 0.4 and 7.3 ± 0.6 g/kg BW/24 h, respectively, in the Deprivation group, the Deprivation + Stress + Vehicle group, the Deprivation + Stress + 1.0 mg/kg Prazosin group and the Deprivation + Stress + 1.5 mg/kg Prazosin group. The alcohol intakes were not significantly different among the five treatment groups ($P = 0.11$). The daily 24-h alcohol intakes during days 11–15 were not significantly correlated with either anxiety-like behavior or the Composite Anxiety Index score within any individual treatment group of 6–7 rats.

Rats treated with vehicle tended to struggle frequently throughout the 1-h restraint stress. In contrast, the prazosin-treated rats tended to lie quietly within ~15 min after onset of restraint and for the remainder of the 1-h trial, without struggling but with their eyes open and with regular breathing. Although times of struggling were not quantitated, the subjectively observed differences between vehicle vs prazosin treatments were striking and consistent. Differences in struggling were not obvious between rats receiving 1.0 vs 1.5 mg/kg doses of prazosin. Rats in all groups were active immediately after release from the restraint tubes.

Results of approach/avoidance testing during the final alcohol deprivation are presented in Fig. 2. Rats that had been stressed at the start of the previous two alcohol deprivations and did not receive prazosin treatment [Deprivation + Stress + Vehicle] exhibited decreased social approach behavior during the third (final) deprivation, compared with Control rats ($P = 0.05$) and compared

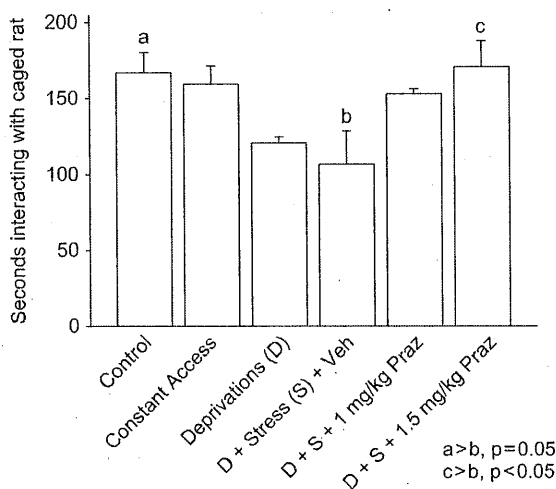


Fig. 2. Social Approach/Avoidance Test. Rats that had been stressed at the start of the first two alcohol deprivations (D + Stress + Veh) exhibited increased anxiety-like behavior (i.e. decreased social approach) in the social approach/avoidance test during the third (final) deprivation, compared with Control rats ($P = 0.05$). Prazosin (1.5 mg/kg) treatment before each of the two stresses blocked this stress effect ($P < 0.05$). Lower dose prazosin (1 mg/kg) treatment before each of the two stresses tended ($P = 0.09$) to decrease the stress effect. Each bar represents the mean + SEM of 6–7 rats/group.

with rats with continuous alcohol access (constant access; trend at $P < 0.06$). Prazosin (1.5 mg/kg) treatment before both episodes of stress blocked this stress response ($P < 0.05$). There was a trend ($P = 0.09$) for the lower dose of prazosin to also decrease this stress response. There was not a significant difference among the groups with respect to time investigating the novel empty cage.

Results of elevated plus-maze testing are presented in Fig. 3. Rats that had been stressed at the start of the first two alcohol deprivations and that did not receive prazosin treatment [Deprivation + Stress + Vehicle] exhibited decreased open arm time (left panel, $P < 0.05$) and decreased open arm entries (right panel, $P < 0.01$) during the third deprivation, compared with Control rats. Treatment with either dose of prazosin (1.0 or 1.5 mg/kg) before each of the stresses in the first two deprivations blocked this stress response. Total arm entries were not significantly different among treatment groups.

We also expressed the results of the sequential social approach/avoidance and the elevated plus-maze tests as a single composite score. Scores in the social approach/avoidance test (one score for each rat: number of seconds in social approach/interaction) were ranked for all rats and a tertile split was used to assign an anxiety score (range 1–3 in whole increments) to each rat: the third that spent the most time in social approach/interaction (considered to reflect least anxiety) received a score of 1; the middle third received a score of 2; and those that spent the least time in social approach/interaction (considered to reflect the greatest anxiety) received a score of 3. Elevated plus-maze behavior was similarly ranked and scored, with the third that spent the most time on the open arms (considered to reflect the least anxiety) receiving a score of 1 and the third that spent the least time on the open arms receiving a score of 3. These scores on the two behavioral tests were summed for each rat, yielding a single composite index ranging from 2 to 6, with increasing values considered to reflect increasing expression of anxiety. As illustrated in Fig. 4, rats that had been stressed at the start of each of the first two alcohol deprivations, without prazosin treatments, exhibited a robust increase in this composite score of anxiety in the subsequent (third) deprivation period when compared with Control rats ($P < 0.01$) or rats that had received constant access to alcohol ($P < 0.01$), as well as a more modest increase ($P \leq 0.05$) when compared with rats that had received alcohol deprivations without administrations of stress. The stress-induced increase in the composite index was eliminated by treatment with prazosin in a dose of 1.0 mg/kg ($P < 0.01$) or 1.5 mg/kg ($P < 0.001$) before each of the two stresses.

DISCUSSION

Restraint stress administered during two consecutive alcohol deprivations in male P rats increased anxiety-like behavior when tested during a third deprivation. This finding is consistent with previous reports that repeated alcohol withdrawals increase subsequent deprivation-induced anxiety behavior in rats, and that this response is facilitated by exposures to stress (reviewed by Breese *et al.*, 2005a). Breese *et al.* (2005a) suggested that repeated withdrawals from alcohol, together with exposure to stressors, sensitize the anxiety response to alcohol withdrawal (similar to multiple withdrawal-induced 'kindling' of seizure activity) and that this increase in anxiety reflects a persisting neuroadaptive change. Facilitation of anxiety by repeated alcohol deprivations, enhanced by stress, is consistent with characteristics of alcoholism, which include increased intensity of withdrawal symptoms and negative affect during abstinence after repeated withdrawal episodes, and is thought to increase the likelihood of alcohol relapse (Koob and LeMoal, 1997).

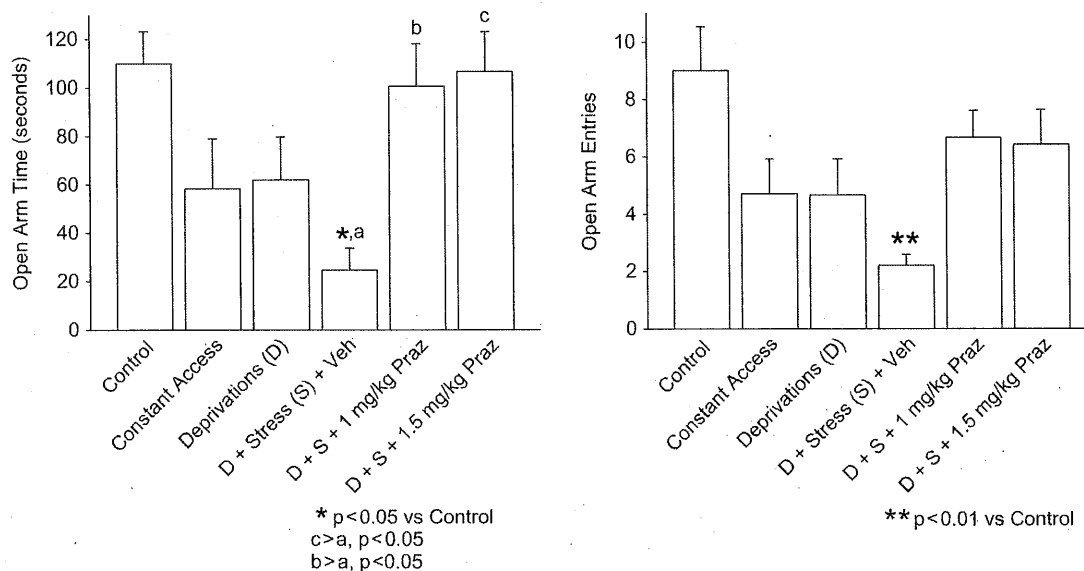


Fig. 3. Elevated plus-maze test. Rats that had been stressed at the start of the first two alcohol deprivations (D + Stress + Veh) exhibited increased anxiety-like behavior (i.e. decreased open arm time [left panel] and decreased open arm entries [right panel]) in the elevated plus-maze test during the third (final) deprivation, compared with Control rats. Treatment with either dose of prazosin (1 or 1.5 mg/kg) before each of the two stresses in the first two deprivations blocked this stress effect. Each bar represents the mean + SEM of 6–7 rats/group.

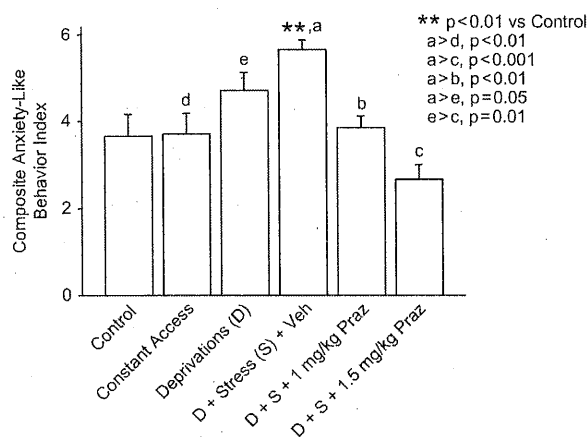


Fig. 4. Composite anxiety-like behavior score. When anxiety-like behavior was expressed as a composite of the results of social interaction and elevated plus-maze tests, with increasing values reflecting increasing anxiety-like behavior (as described in Results), rats that had been stressed at the start of the first two alcohol deprivations exhibited robust increases ($P < 0.01$) in anxiety-like behavior in the subsequent (final) deprivation when compared with Control rats and with rats that had received constant access to alcohol, as well as more modest increases ($P = 0.05$) when compared with rats that had received alcohol deprivations without added stresses. This [deprivation + stress] induced increase in the composite index of anxiety-like behavior was suppressed by treatment with prazosin 1 mg/kg and prazosin 1.5 mg/kg ($P < 0.01$ and $P < 0.001$, respectively) before each of the stresses in the first two deprivations, compared with the [deprivation + stress + vehicle] rats. Each bar represents the mean + SEM of 6–7 rats/group.

Prazosin administration before stress during two alcohol deprivations reduced anxiety-like behavior during a third deprivation in the current study, suggesting that decreasing α_1 -adrenergic receptor-mediated signaling when encountering stress during alcohol deprivation may prevent the persistent neuroadaptation that Breese *et al.* (2005a) suggested to be responsible for increased anxiety during alcohol abstinence.

Previous studies using the repeated alcohol deprivation-induced anxiety-like behavioral model in unselected (outbred) rats have demonstrated that drugs which alter benzodiazepine receptor-, serotonin receptor-, CRF type 1 receptor (CRF1)- or less specific mixed receptor-mediated mechanisms can each prevent the increase in abstinence-induced anxiety-like behavior during subsequent alcohol deprivation, suggesting that multiple systems contribute to the facilitation of deprivation-induced anxiety (Breese *et al.*, 2005a,b). However, in P rats the specific blockade of CRF₁ during alcohol deprivation was the most effective treatment for blocking this facilitation of deprivation-induced anxiety behavior (Overstreet *et al.*, 2005). In the current study with P rats, specific blockade of α_1 -adrenergic receptors by prazosin prior to stress during two alcohol deprivations was likewise highly effective in decreasing anxiety-like behavior during a third deprivation. This suggests contributions of two pathways (noradrenergic- and CRF₁-mediated), or an integrated pathway, in mediating the facilitation of deprivation-induced anxiety in P rats. Stress increases both noradrenergic and CRF-mediated responses (Koob, 1999); forebrain noradrenergic neurons provide stimulatory innervation of CRF neurons (Yang *et al.*, 1990; Itoi *et al.*, 1994); CRF neurons conversely provide stimulatory innervation of forebrain noradrenergic neurons (Koob, 1999; Jedema and Grace, 2004); and prazosin appears to disrupt a feed-forward loop of

extra-hypothalamic CRF-noradrenergic interactions (Yang *et al.*, 1990; Feldman and Weidenfeld, 1998; Koob, 1999; Ryabinin *et al.*, 2002). Consequently, blocking signaling by either CRF₁- or α 1-adrenergic-mediated mechanisms could potentially block the effects of stress on a noradrenergic-CRF feed-forward loop that has been suggested to mediate increased anxiety during alcohol withdrawal and abstinence (Koob, 1999; Koob and LeMoal, 1997).

Although sympathetic activation is increased during alcohol deprivation in rats and humans (Ehrenreich *et al.*, 1997; Patkar *et al.*, 2004; Rasmussen *et al.*, 2006) and excessive brain and peripheral noradrenergic activation are associated with increased anxiety (Sullivan *et al.*, 1999), it remains to be determined how acutely blocking noradrenergic signaling (including potential interactions between noradrenergic and CRF signaling) at the time of stress suppresses anxiety-like behavior during a subsequent alcohol deprivation 7 days later. It has been demonstrated that administrations of corticosterone instead of stress do not decrease subsequent alcohol deprivation-induced anxiety in this model (Breese *et al.*, 2004), so it is unlikely that prazosin effects on corticosterone secretion play an important mediating role. However, stimulation of noradrenergic activation enhances, and blockade reduces, memory for material with emotional impact (O'Carroll *et al.*, 1999), so prazosin treatment could potentially decrease stress-enhanced increases of alcohol deprivation-induced anxiety by decreasing memory of the stress. Another possibility is that anxiolytic effects of prazosin (Skelly and Weiner, 2014) may have decreased the perceived stressfulness of the stressor, consistent with subjective evidence in the current study suggesting that rats treated with prazosin struggled less and appeared to relax more quickly during the 1-h restraint. A potentially related mechanism is suggested by evidence that pretreatment with prazosin, at the same dose and time of administration as those used in the current study, reduces 1 h restraint stress-induced immediate early gene expression in several telencephalic, diencephalic and brainstem areas of the rodent brain, without altering motor coordination (Stone and Zhang, 1995).

Alcohol intakes during the final 5 days before determination of anxiety-like behavior were not significantly different among the treatment groups, although there was a modest trend ($P = 0.011$)—with least intake by rats receiving continuous alcohol access and most intake by rats receiving Deprivation + Stress + Vehicle treatment. This trend is consistent with changes in alcohol intake previously reported for P rats in this model (Overstreet *et al.*, 2005), although the small sample size in the current study may have been insufficient to demonstrate these anticipated changes. The final 5 days of alcohol intake within each treatment group was also not significantly correlated with behavioral testing results during the subsequent alcohol deprivation, although correlational analyses based on $n = 5$ –6 do not allow meaningful interpretation.

The current results demonstrate that treatment with prazosin, when confined to only the time that stress is encountered during alcohol deprivations, blocks the increase in anxiety-like behavior that is normally seen during subsequent alcohol deprivation. This effect was seen during alcohol deprivation 7 days after the most recent stressor and prazosin treatment. We speculate that prazosin might decrease alcohol drinking through two distinct mechanisms, i.e. an acute effect that is seen when prazosin is administered at the time of alcohol intake, as we have previously reported, and also by suppressing stress-induced increased anxiety during subsequent alcohol deprivation. Acute prazosin-induced suppression of alcohol drinking, together with prazosin's suppression of anxiety during subsequent alcohol deprivation (abstinence), could potentially both

contribute to a reduction in alcohol consumption during the repeated cycles of alcohol drinking and relapse that are experienced by many individuals with alcoholism. These results also suggest that using prazosin on an 'as needed' basis when stressful events are anticipated during abstinence could be of potential benefit to alcoholic individuals who are trying to avoid relapse. Further studies that incorporate direct measures of alcohol drinking are warranted to test these potential mechanisms and treatments.

FUNDING

Supported by resources from VA Puget Sound Health Care System Mental Health Service, VISN 20 MIRECC and National Institutes of Health Grants AA018604 and AA017839 (Rasmussen); AA018604 and AA007611 (Froehlich).

CONFLICT OF INTEREST STATEMENT

None declared.

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cal pathways involved in sexual desire and function. This paper will present preliminary data suggesting that known neuroendocrine correlates of PTSD are associated with SD. In combat veterans with PTSD ($n = 76$), loss of sexual interest was associated with lower levels of the adrenal androgen DHEA ($\beta = .316$, $t_{72} = -2.364$, $p = .021$) and plasma cortisol ($\beta = -.378$, $t_{72} = -3.344$, $p = .001$), and with an attenuated response to the dexamethasone suppression test ($\beta = -.314$, $t_{67} = -2.565$, $p = .013$). Heightened catecholamines as reflected by the NE/cortisol ratio were associated with problems during sexual relations ($r = .382$, $p = .028$, $df = 31$) and also predicted PTSD intrusive symptoms ($\beta = .216$, $t_{89} = 2.04$, $p = .044$). In a separate study of treatment seeking veterans with PTSD, urinary NE was associated with difficulty achieving orgasm ($\beta = .340$, $t_{39} = 2.34$, $p = .024$). In a subsample analysis, testosterone levels did not distinguish SD, suggesting that sexual problems were not the result of organic disorder. Implications and avenues for future research will be discussed.

<http://dx.doi.org/10.1016/j.psyneuen.2015.07.533>

PO86

HPA axis dysregulation in patients with hypersexual disorder

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Recent focus has been raised on hypersexual disorder that was suggested as a diagnosis for the DSM-5. However, little is known about the neurobiology behind this disorder. A dysregulation of the hypothalamic pituitary adrenal (HPA) axis has been shown in psychiatric disorders but it is not investigated in hypersexual disorder. The aim of this study was to investigate the function of the HPA axis in hypersexual disorder.

The study includes 67 male patients with hypersexual disorder and 39 healthy male volunteers. Basal morning plasma levels of cortisol and ACTH were assessed and low dose (0.5 mg) dexamethasone suppression test was performed with cortisol and ACTH measured post dexamethasone administration. Non-suppression status was defined with DST-cortisol levels ≥ 138 nmol/l. The Sexual Compulsive scale (SCS), Hypersexual disorder current assessment scale (HD:CAS), Montgomery-Åsberg Depression Scale-self rating (MADRS-S) and Childhood trauma questionnaire (CTQ), were used for assessing hypersexual behavior, depression severity and early life adversity.

Patients with hypersexual disorder were significantly more often DST non-suppressors, had significantly higher DST-ACTH levels and their DST-Cortisol levels showed a trend to be higher compared to healthy volunteers. The patients reported significantly more childhood trauma and depression symptoms compared to healthy volunteers. CTQ scores showed a significant negative correlation with DST-ACTH whereas SCS and HD:CAS scores showed a negative correlation with baseline cortisol in hypersexual patients. The diagnosis of hypersexual disorder was significantly associated with higher plasma DST-ACTH even when adjusted for childhood trauma.

The results suggest a possible HPA axis dysregulation in patients with hypersexual disorder.

<http://dx.doi.org/10.1016/j.psyneuen.2015.07.534>

PO87

Reduction of $\alpha 1$ -adrenergic signaling at the time of traumatic stress prevents subsequent development of increased alcohol drinking

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Activation of $\alpha 1$ -adrenergic receptor (AR)-mediated mechanisms contributes to post-traumatic stress disorder (PTSD) and to increased acoustic startle response. Pre-stress acoustic startle response (ASR) predicts subsequent increased startle response in a rat PTSD model in which a single traumatic stress (TS, 10 second footshock) is followed by weekly contextual reminders (R) of the TS. ASR also predicts development of increased alcohol drinking in a rat intermittent alcohol access (IAA) model (20% alcohol access for 24 h/day on 3 non-consecutive days/week). We thus hypothesized that reduction of $\alpha 1$ -AR signaling at the time of TS would prevent subsequent development of the increased alcohol drinking that commonly accompanies development of PTSD. Male Wistar rats received either TS or no-shock (NS). After 4 weekly R, IAA was provided for 4 weeks. During the final week, average alcohol intake in the first hour of each IAA was increased ($p < 0.05$) in [TS+R] vs [NS+R] rats. The $\alpha 1$ -AR antagonist, prazosin (PRZ; 1.5 mg/kg), administered IP at the time of the single TS decreased the [TS+R]-induced IAA alcohol drinking ($p < 0.05$, relative to treatment with vehicle), as did PRZ+the β -AR antagonist, propranolol (PRO, 5 mg/kg). These results suggest that PRZ or PRZ+PRO treatment at the time of TS can prevent subsequent development of increased alcohol drinking. We will further address effects of PRZ or PRZ+PRO administered at other time points relative to TS and R, and potential roles of corticosterone. Supported by VA Puget Sound Health Care System, Seattle, Washington and by US Army Medical Research CDMRP W81XWH-13-1-0126.

<http://dx.doi.org/10.1016/j.psyneuen.2015.07.535>

PO88

Is salivary estriol detectable in very early pregnancy?

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Background: Estrinol is produced in large quantities by the placenta during pregnancy and is important for early pregnancy maintenance. In maternal blood, estrinol can be detected from the 8th week of pregnancy and increases sharply after the 10th week under the influence of the HPA axis hormone ACTH. Although estrinol can be reliably analyzed in saliva from the 13th week, informa-

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REDUCTION OF α 1- AND β -ADRENERGIC SIGNALING AT THE TIME OF TRAUMATIC STRESS REDUCES SUBSEQUENT DEVELOPMENT OF INCREASED ALCOHOL DRINKING IN RATS
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We previously demonstrated that pre-stress acoustic startle response (ASR) predicts subsequent further increased ASR in a rat PTSD model in which a single traumatic stress (TS) is followed by repeated weekly contextual reminders (R) of the TS. We also demonstrated that ASR predicts subsequent development of increased voluntary alcohol drinking in rats. Since noradrenergic activation contributes to increased ASR, anxiety, alcohol drinking, and PTSD, we hypothesized that reduction of noradrenergic signaling at the time of TS would decrease the subsequent development of increased alcohol drinking that commonly accompanies development of PTSD and increased anxiety. Male Wistar rats received either TS (10 sec footshock) or no-shock (NS). After 4 weekly R, intermittent alcohol access (IAA; choice between water and 20% alcohol for 24 h/day on 3 non-consecutive days/week) was provided for 4 weeks. Combined IP administration of the α 1-adrenergic antagonist, prazosin (PRZ, 1.5 mg/kg), and the β -adrenergic antagonist, propranolol (PRO, 5 mg/kg), at the time of the single 10 sec TS decreased average alcohol intake by 46% in the first hour of each IAA, 8-12 weeks after the TS ($p < 0.001$, relative to treatment with vehicle). Administration of PRZ (1.5 mg/kg) alone at the time of the single TS also decreased average alcohol intake ($p < 0.001$, relative to vehicle), but only by 32%, which was less than the suppression by [PRZ+PRO] ($p < 0.01$). Alcohol preference was likewise suppressed more by [PRZ+PRO] than by PRZ alone ($p < 0.01$). Trauma memory testing before the IAA was initiated at 8 weeks after the single TS confirmed that the TS-R rats remembered the single TS and that the context of the TS remained aversive (expressed as increased avoidance behavior and increased defecation during R, each $p < 0.01$ relative to NS). These results suggest that PRZ treatment at the time of a TS can decrease subsequent development of increased voluntary alcohol drinking 8-12 weeks after TS, that this response to PRZ is enhanced by co-treatment with PRO, and that these responses are not mediated by decreasing the perceived aversiveness of the TS or by decreasing memory of the TS. PRZ or [PRZ+PRO] administration at the time of TS potentially may provide effective preventive treatment for TS-induced development of alcohol abuse and -perhaps- PTSD or other correlates of PTSD. Further investigations are addressing effects of treatments at other time points relative to TS and R, resolution of responses by TS-sensitive versus -resilient subjects, and mechanisms. Supported by VA Puget Sound Health Care System, Seattle, WA and by US Army Medical Research CDMRP W81XWH-13-1-0126.

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IMPACT OF CHRONIC ETHANOL SELF-ADMINISTRATION ON KAPPA OPIOID RECEPTOR REGULATION OF DOPAMINE SIGNALING IN NONHUMAN PRIMATES

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Although alcoholism is one of the most prevalent disorders in the United States, with over 18 million individuals meeting the criteria for an alcohol use disorder, the exact neurobiological bases of this condition remain obscure. Recently, it has been demonstrated that kappa-opioid receptor (KOR) signaling in the striatum plays a critical role in the increased reinforcing efficacy of ethanol following ethanol vapor exposure in rodent models. Here we examined the effects of chronic voluntary ethanol self-administration in macaques on dopamine neurotransmission and the ability of KORs to regulate dopamine release in the nucleus accumbens core. Three cohorts of nonhuman primates were given free access to 4% ethanol (w/v) for 22 h/day. These cohorts were composed of male cynomolgus, female rhesus or male rhesus macaques, and were given access to ethanol for 6, 12, or 18 months, respectively. Ex vivo fast-scan cyclic voltammetry was then conducted the nucleus accumbens core to determine dopamine signaling kinetics as well as the ability of U50,488 (KOR agonist) to inhibit dopamine release. We found that chronic ethanol drinking increased dopamine uptake rates, which could have implications for reductions in basal dopamine tone in vivo during ethanol withdrawal. Further, across sex, strain and exposure length, ethanol use augmented the ability of KORs to inhibit dopamine release, demonstrating that ethanol-induced increases in KOR sensitivity are widespread and independent of other factors. Finally, KOR sensitivity was positively correlated with lifetime ethanol intake, suggesting that changes in KOR regulation of dopamine release may be a determinant of aberrant drinking behaviors. Nonhuman primate models of ethanol abuse represent a highly translational avenue for identifying molecular targets for pharmacotherapeutic compounds, and here we show, for the first time, that voluntary ethanol self-administration has a unique effect on KOR sensitivity and regulation of dopamine release directly at the dopamine terminal that was positively correlated with drinking behavior. Together, these data provide novel insight into ethanol-induced dysregulation of opioid signaling and suggest that dopaminergic dysfunction may be mediating increases in voluntary drinking. Importantly, KOR antagonists may be efficacious in reducing drinking behaviors in alcoholics.

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TAAR1 ACTIVATION PREFERENTIALLY DECREASES PHASIC DOPAMINE RELEASE AND DECLINES ETHANOL SEEKING BEHAVIOR IN RATS

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Recent studies suggest that trace amine-associated receptor 1 (TAAR1) may represent a novel therapeutic target for the treatment of several neuropsychiatric disorders, including addiction. However, the mechanisms involved in the psychotropic actions of TAAR1 agonists are still unclear. The present study explored the effects of a partial TAAR1 agonist, RO5263397, on presynaptic dopamine (DA) transmission, using optogenetics combined with real-time DA detection. We also assessed the effect of this compound on operant ethanol drinking behavior. We applied a new viral technology to restrict the expression of ChR2 to DA cells in the VTA of Long Evans rats, driving ChR2-EYFP expression via a tyrosine hydroxylase promoter. The viral construct was microinjected into the VTA of rats ($n = 5$) and DA release was measured by fast-scan cyclic voltammetry in anesthetized rats. Importantly, the level of ChR2 expression was sufficient to allow us to optogenetically mimic tonic and phasic patterns of accumbal DA release. The experiments indicated a significant effect of RO5263397 (10 mg/kg, i.p.) on the amplitude of the optogenetically-induced DA signal ($p < 0.0001$). Notably, the compound differentially affected phasic and tonic patterns of DA release. Thus, the drug decreased phasic DA release more powerfully than tonic DA efflux ($p < 0.05$). Since there are marked regional variations in DA dynamics between striatal and accumbal terminals, we explored local effects of RO5263397 on DA efflux in the nucleus accumbens core, shell and dorsal striatum. The study was performed on brain slice preparations, where a single pulse (4 ms) of electrical stimulation was applied to evoke DA transients. High concentrations of the compound significantly decreased DA release in the nucleus accumbens core ($p < 0.05$ for 50 μ M and $p < 0.0001$ for 100 μ M) and dorsal striatum ($p < 0.0001$ for 100 μ M). There was a significant difference in the effects of the compound at 50 and 100 μ M between regions ($p < 0.05$). Specifically, electrically-evoked DA release in the shell region was less responsive to RO5263397 application. These in vitro data suggest that RO5263397 at high concentrations can affect presynaptic DA receptors at the level of terminals (preferentially, in the nucleus accumbens core and dorsal striatum). Finally, the effects of the compound on ethanol drinking behaviors were studied using the operant self-administration procedure. The rats were trained to press a lever 30 times daily for 20 min access to 10% ethanol. After subjects displayed stable appetitive and consummatory behaviors (≈ 6 weeks), the effect of RO5263397 (5 or 10 mg/kg, i.p.) or saline were evaluated. Pretreatment with the TAAR1 agonist significantly suppressed lever press behaviors at both doses tested with a main effect of treatment ($p < 0.001$). Importantly, when access to ethanol was provided, no significant changes in consummatory measures (e.g. number of licks, ethanol intake) were observed. In conclusion, these results suggest that the TAAR1 agonist tested selectively suppresses ethanol seeking behaviors, presumably through the inhibition of phasic DA release in the nucleus accumbens. This proposes the fascinating possibility that TAAR1 agonists may be considered as promising candidates for the treatment of alcohol addiction.

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DHM EFFECTS ON ETHANOL-INDUCED DOPAMINE RELEASE IN THE RAT NUCLEUS ACCUMBENS

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Direct and indirect involvement of the inhibitory GABA-A and glycine receptors has previously been demonstrated to be of importance for the ability of ethanol (EtOH) to increase dopamine levels in the nucleus accumbens (nAc). In a series of studies, we suggested that EtOH primarily acts in the nAc, where it triggers a neuronal circuitry involving nAc glycine receptors as well as ventral tegmental nicotinic acetylcholine receptors, in order to elevate extracellular accumbal dopamine. We have also investigated a number of substances known to decrease EtOH intake in rodents and/or humans, alone or in combination with EtOH with regards to influence on nAc dopamine. In the present study we aimed to explore the influence of dihydromyricetin (DHM) alone and in combination with EtOH on extracellular levels of dopamine in the nAc, as measured by in vivo microdialysis in freely moving Wistar rats. Since EtOH previously was demonstrated to increase endogenous levels of the amino acid taurine, a ligand to both GABA-A and glycine receptors, and DHM previously was suggested to mediate its EtOH reducing effects via the GABA-A receptor we also measured taurine, glycine, serine and glutamate in the dialysate samples. After systemic administration of DHM we found a modest (approximately 20%) increase of nAc dopamine whereas no alteration of any of the measured amino acids was detected. In rats pretreated with DHM a systemic injection of EtOH (2.5 g/kg i.p. 20 min after DHM) failed to produce any further dopamine elevation. In the present study DHM, a substance with potential interest for treating human alcohol use disorders, was found to display a similar pattern with regards to nAc dopamine as previously found for several other investigated compounds (acamprosate, varenicline and the glycine uptake inhibitor Org-25935). Thus, since yet another compound with affinity for inhibitory receptors in the nAc was demonstrated to produce a modest dopamine increase and prevent EtOH from further increasing extracellular dopamine levels we suggest that this drug profile is beneficial in the purpose of controlling excessive EtOH intake in humans.

ABSTRACTS OF ORAL PRESENTATIONS

TUESDAY, JUNE 27

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PUTTING IMPLICIT ALCOHOL COGNITIONS TO THE TEST IN PERSONALITY, CLINICAL, AND DEVELOPMENTAL MODELS OF ALCOHOL RISK

ORGANIZER/CHAIR: ROISIN O'CONNOR

RATIONALE AND CONTENT: Psychosocial cognitive theories have long pointed to the role of memory associations in predicting risk for alcohol misuse. For example, if one learns that alcohol enhances positive mood or takes the edge off, then drinking to regulate internal states is likely. Through extensive empirical tests and revisions to and integration of theories, the nuances of problematic drinking consistently emerged and presented a conceptual challenge. While these models accounted for the effects of positive alcohol memory associations (*alcohol makes me feel good*) on alcohol use, they did not address the negative expectancies (*alcohol is ruining my marriage*) that risky drinkers likely hold, and which are important determinants of drinking. Delayed discounting theories offer some resolution, taking into account the relative value of both immediate positive effects (reward) and delayed negative effects (MacKillop et al., 2011). However, the mechanistic cognitive process is not well accounted for in these theories. Dual cognitive process models offer a unifying framework (Chaiken & Trope, 1999), suggesting that two modes of cognitive processing influence alcohol use. These are: (1) a reflective, deliberate process that engages top down reasoning to arrive at a thoughtful decision; (2) a reflexive process that is automatically activated by internal or environmental cues. The former is often referred to as a self-regulatory or controlled process and is typically measured using explicit self-reports. The second is often referred to as an impulsive, implicit, or automatic process and is typically measured using computer-administered reaction time assessments [Implicit Association Test (Greenwald et al., 1995) and its variants]. These models suggest that alcohol use is determined by the interplay of these processes (Wiers et al., 2007, 2010). The self-regulatory process, which permits thoughtful decisions about the cons of drinking, can be diminished by state affect, extensive alcohol abuse, and environmental triggers. With inhibited control, implicit alcohol cognitions take precedence. Thus, implicit cognitions that favour alcohol use are thought to be pivotal to alcohol risk. The goal of this symposium is to present recent findings that put this theory to the test. The link between implicit alcohol cognitions and drinking behavior has been well studied. Some of this work comes from the labs of the presenters and discussant for this symposium. This work is important and has supported the distinction of explicit and implicit alcohol cognitions. However, it is time to challenge these models further, putting them to the test by integrating them within personality, clinical, and developmental models of alcohol misuse risk. Our driving question is whether implicit alcohol cognitions perform as expected, thereby providing clarity to risk models. We will respond to this question by presenting results from experimental and prospective studies. In the first set of talks, Dr. Read (Talk 1) and Dr. O'Connor (Talk 2) will present work from experimental studies that used cue exposure and mood manipulations to push implicit cognitions around in the lab. In the second set of talks, Dr. Colder (Talk 3) and Dr. Keough (Talk 4) will present findings from two formative developmental periods, these include early-to-late adolescence and the transition out of university. Our discussant, Dr. Wiers, who has made substantial contributions to the dual process and alcohol use literature will help to pull these studies together, pointing towards next steps in theoretical and etiological alcohol use model development.

INTRODUCTION - ROISIN M O'CONNOR

USING THE IAT TO EXAMINE ALCOHOL COGNITIONS IN PTSD AND TRAUMA CUE EXPOSURE - JENNIFER READ - Dr. Read

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will present a lab-based study that tests the mechanistic role of implicit cognitions in PTSD risk for alcohol misuse. This work investigates trauma cue effects on implicit alcohol cognitions within clinical PTSD and non-clinical samples. The results suggest that emotionally laden cues bring positive alcohol (implicit) associations to the forefront, thus helping to explain how trauma may trigger alcohol use.

SOCIAL ANXIETY RELATED SHIFTS IN IMPLICIT COGNITION FOLLOWING ALCOHOL INITIATION IN THE LAB - ROISIN O'CONNOR

Dr. O'Connor will present a study examining how social anxiety-related implicit alcohol cognitions unfold from pre-to-post alcohol initiation in the lab. Results suggest that a priming dose of alcohol promotes activation of tension reduction alcohol cognitions for those high in social anxiety. This work points to in-the-moment shifts in implicit cognition that might help to explain social anxiety risk for alcohol misuse.

SENSITIVITY TO REWARD AND PUNISHMENT SHAPE IMPLICIT ALCOHOL ATTITUDES IN ADOLESCENCE - CRAIG COLDER

Dr. Colder will present a prospective study that examines the effect of individual differences on the shaping of implicit alcohol cognitions during the transition from early-to-late adolescence. Results of this seven-year study align with theory, in that youth motivated by reward (who were less responsive to punishment) showed a weakening of negative implicit alcohol cognitions across time.

EXAMINING IMPULSIVE AND SELF-REGULATORY COGNITIVE PROCESSES AS PREDICTORS OF MATURING OUT OF ALCOHOL MISUSE AMONG YOUNG ADULTS - MATTHEW KEOUGH

Dr. Keough will present a study that examines the effect of implicit and self-regulatory cognitive processes on the maturing out (of problematic drinking) phenomenon we see post-university. Results of this one-year prospective study suggest that while negative implicit alcohol cognitions coincide with maturing out, self-regulatory processes may overcome this bias and promote risk. This interplay of cognitive processes fits with theory.

DISCUSSANT/QUESTION MODERATOR - REINOUT WIERS - Dr. Wiers will frame the discussion around the application of contemporary dual process models of addiction within developmental and clinical contexts. He will link these studies to extant empirical evidence and highlight next steps for this line of query.

THE NEGLECTED CATECHOLAMINE: NORADRENERGIC MECHANISMS IN REGULATION OF ALCOHOL-RELATED BEHAVIORS

ORGANIZER/CHAIR: RUEBEN GONZALES

ORGANIZER: DENNIS RASMUSSEN

RATIONALE AND CONTENT: Little attention has been given over the years to the potential role of norepinephrine in the mechanism of action of alcohol, especially compared to dopamine, a related neurotransmitter as well as the biosynthetic precursor of norepinephrine in neurons. However, recent work has sparked new interest in norepinephrine systems as a target for alcohol's central actions. Norepinephrine is involved in cognition, attention, and stress responses, all of which may contribute to behavioral effects of acute and chronic ethanol. This symposium brings together basic and clinical researchers to present recent findings that support the notion that norepinephrine pathways in the brain are altered by ethanol and that adrenergic drugs may be useful for treatment of alcohol use disorders.

This symposium abstract also is reported at Alcohol. Clin. Exp. Res. 41 (S1): 13A, 2017

ABSTRACTS OF ORAL PRESENTATIONS

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INTRODUCTION - LORENZO LEGGIO

CHRONIC STRESS AND ETHANOL-MEDIATED ALTERATIONS IN NOREPINEPHRINE TRANSMISSION IN MESOLIMBIC CIRCUITRY - ANUSHREE KARKHANIS - Several pre-clinical studies have shown that rodents exposed to ethanol chronically lead to dependence and escalated intake of ethanol. Similarly, rodents exposed to chronic stress show significantly greater ethanol intake compared to their unstressed controls, thus showing a phenotype of increased vulnerability to develop alcohol use disorder. It is known that norepinephrine is modulated by exposure to stress and mediates anxiety-like behavior. The effects of chronic stress and chronic ethanol exposure on norepinephrine transmission in the basolateral amygdala (BLA) and the nucleus accumbens (NAc) were studied. Chronic stress results in ethanol (1 g/kg)-induced norepinephrine release compared to a lack of ethanol effect in controls in the NAc. Furthermore, an augmented norepinephrine response to a higher dose of ethanol (2 g/kg) was observed in chronic stress exposed ethanol naïve rats in both regions. Animals exposed to chronic ethanol vapor showed greater norepinephrine in the BLA at baseline compared to air-exposed controls. A pharmacological stressor resulted in an enhanced norepinephrine response in ethanol-exposed animals. These results imply that the norepinephrine system is primed and stimulation of this system results in a sensitized response in animals exposed to chronic stress and/or ethanol.

NOREPINEPHRINE RESPONSE TO ACUTE ADMINISTRATION OF ETHANOL AND AFTER ETHANOL SELF-ADMINISTRATION IN THE MEDIAL PREFRONTAL CORTEX - ASHLEY VENA

The pharmacological effects of ethanol on extracellular norepinephrine concentrations in the medial prefrontal cortex following two different routes of ethanol administration were determined. An acute intravenous infusion of ethanol stimulated dialysate norepinephrine concentrations to 77% over baseline, while saline infusion did not enhance the response. Microdialysis was also performed in rats during an operant self-administration session. Rats experienced about one week of self-administration of a sweetened ethanol solution (10S10E) or a sucrose solution (10S). A separate control group did not receive any drinking solutions in the operant chambers. Basal norepinephrine concentrations were significantly different among the three groups: 0.40 ± 0.06 nM in the 10S10E group, 0.55 ± 0.01 nM in the handling group, and 0.62 ± 0.07 nM in the 10S group. During the operant session, a transient spike in norepinephrine was observed during the transfer from the home cage to the operant chamber that lasted for the duration of the wait period in all three groups. The results show that ethanol exerts direct pharmacological actions on central noradrenergic neurons. Furthermore, limited voluntary ethanol consumption appears to be sufficient to alter tonic norepinephrine signaling in the medial prefrontal cortex.

VARIABILITY IN ALCOHOL DRINKING RESPONSES TO SUPPRESSION OF NORADRENERGIC SIGNALING IN OUTBRED RATS - DENNIS RASMUSSEN

Voluntary alcohol drinking in selectively-bred alcohol-preferring (P) rats is suppressed by administration of the α_1 adrenergic antagonists, prazosin or doxazosin. This suppression by prazosin is consistent among diverse experimental conditions and is increased when combined with the β -adrenergic antagonist, propranolol. However, selectively-bred P rats do not exhibit the genetic and behavioral diversity characteristic of outbred rats or humans, and responses to prazosin and doxazosin have been demonstrated to vary among individuals within clinical studies. Dr. Rasmussen will examine responses to prazosin in outbred Wistar rats that likewise exhibit variable alcohol drinking responses that are dependent upon gender, individual behavioral characteristics, and alcohol exposure history. He will also present results from a study with Wistar rats in a model of

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PTSD combined with intermittent alcohol access, in which suppression of alcohol drinking by either prazosin or prazosin+propranolol ($p < 0.00$ for each) was dependent upon history of traumatic stress. The variability in voluntary alcohol drinking responses in outbred rat models may inform clinical studies with the goal of prospectively identifying which patient subpopulations are most likely to decrease alcohol drinking in response to suppression of noradrenergic signaling.

THE ALPHA-1 BLOCKADE BY DOXAZOSIN IN REGULATION OF ALCOHOL DRINKING IN ALCOHOL-DEPENDENT PATIENTS - CAROLINA HAASS-KOFFLER - Evidence suggests that α_1 blocker prazosin reduces alcohol drinking in alcohol-dependent (AD) individuals, and pre-treatment blood pressure (BP) predicts therapeutic response in PTSD patients. The α_1 blocker doxazosin has pharmacokinetic advantages over prazosin, but has never been studied for AD. Dr. Haass-Koffler will present the data from a double-blind placebo-controlled randomized clinical trial (RCT) conducted in AD individuals seeking outpatient treatment. Previously reported RCT data shows that doxazosin reduced drink per week [DPW, $p_{corrected} < 0.001$, $d = 1.30$] and heavy drinking days [HDD, $p_{corrected} < 0.001$, $d = 1.30$] in patients with high family history density of alcoholism (FHDA). New unpublished results now shows, with pre-treatment standing diastolic BP as moderator, there were significant BP x medication interaction for DPW [$p < 0.01$, $d = .80$] and HDD [$p = 0.05$, $d = 1.11$]. Post-hoc analyses showed a significant medication effect in patients with high BP in reducing DPW and HDD. Taken together these findings suggest that doxazosin may be effective selectively in AD patients with high FHDA and that higher standing diastolic BP at baseline. The results obtained from this RCT provide evidence for a personalized medicine approach on the use of α_1 blockade to treat AD patients.

DISCUSSANT / QUESTION MODERATOR - RAYE LITTEN

NON-CODING RNAs IN ALCOHOLIC LIVER DISEASE: FROM BIOMARKERS TO TARGETED THERAPEUTICS ORGANIZERS/CHAIRS: SUTHAT LIANGPUNSAKUL AND LI WANG CHAIR: DALE HERELD

RATIONALE AND CONTENT: The new landscape of human transcriptome along with the identification of non-coding RNAs (ncRNAs) has uncovered their importance in the pathophysiology of human diseases. A non-coding RNA (ncRNA) is an RNA molecule that is not translated into a protein. Non-coding RNA genes include highly abundant and functionally important RNAs such as microRNAs and the long non-coding RNAs (lncRNAs). A microRNA (miRNA), a small non-coding RNA molecule containing about 22 nucleotides, functions in RNA silencing and post-transcriptional regulation of gene expression. lncRNAs are a subgroup of ncRNAs with approximately 200 nucleotides. lncRNAs are expressed at lower levels when compared to protein-coding genes. They are known to regulate gene transcription and involve in controlling metabolic processes. Metabolic derangements in fat cell metabolism and adipogenesis, commonly seen in excessive alcohol use subjects, are also under the regulation of lncRNAs. Recent studies have shown the importance of these ncRNAs in the pathogenesis of several liver diseases including alcoholic liver disease. The goal of this symposium is to summarize the roles of ncRNAs from bench, the mechanism and their function in liver diseases, to bedside, the clinical implications in patients with alcoholic liver disease.

INTRODUCTION - DALE HERELD

This oral presentation is also reported at Alcohol. Clin. Exp. Res. 41(S1): 236, 2017

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A SINGLE BRIEF TRAUMATIC STRESS PRODUCES LONG-TERM CHANGES IN NORADRENERGIC REGULATION OF ALCOHOL DRINKING IN MALE WISTAR RATS
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CHRONIC ALCOHOL IMPAIRS METABOTROPIC GLUTAMATE RECEPTOR 2-MEDIATED LONG-TERM DEPRESSION OF EXCITATORY TRANSMISSION IN THE DORSAL STRIATUM
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Chronic alcohol exposure produces a variety of neuroadaptations that contribute to addiction-related behaviors including escalation of alcohol consumption and a transition from goal-directed to habitual alcohol seeking. Recent studies have implicated metabotropic glutamate receptor 2 (mGlu₂) as a potential mediator of synaptic changes that are induced by chronic alcohol. Importantly, loss of mGlu₂ increases alcohol consumption and preference. However, the specific circuit changes that underlie this effect remain unclear. mGlu₂ is a robust modulator of glutamatergic transmission in the dorsal striatum, a critical brain region for the development of addictive behaviors. Using a combination of brain slice electrophysiology and optogenetic techniques, we have characterized the effects of mGlu₂ activation at corticostriatal and thalamostriatal inputs to medium spiny neurons (MSNs), which are the primary projection neurons of the striatum. We have confirmed that the mGlu_{2/3} agonist LY379268 (100 nM) produces a robust (>50%), long-term depression (LTD) of electrically-evoked excitatory postsynaptic currents (EPSCs) in the dorsolateral striatum. Following viral expression of channelrhodopsin-2 (ChR2) in corticostriatal and thalamostriatal projections, LY379268 induces robust LTD of optically-evoked transmission at both inputs. To evaluate the effect of chronic alcohol on mGlu₂-mediated modulation of glutamatergic transmission in the dorsal striatum, we employed a two week chronic intermittent ethanol (CIE) paradigm followed by a brief (3-6 day) withdrawal period. Interestingly, CIE disrupted the long-term effect of LY379268 on electrically-evoked glutamatergic transmission, but had little effect on short-term depression. Conversely, bath application of 40 mM ethanol to slices from naïve mice did not disrupt mGlu₂-LTD. Excitantly, incubation of slices with a positive allosteric modulator (PAM) of mGlu₂ rescued mGlu₂-LTD following CIE, providing support for the idea that behavioral adaptations related to loss of mGlu₂ function in the striatum could be reversed by mGlu₂ PAM treatment. Ongoing studies aim to evaluate the input specificity of the CIE-induced disruption of mGlu₂-mediated synaptic modulation. These studies increase our understanding of mGlu₂-mediated modulation of glutamatergic transmission in the striatum and provide preliminary evidence that chronic alcohol exposure impairs the ability of mGlu₂ to modulate synaptic transmission.

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CO-ADMINISTRATION OF ETHANOL AND NICOTINE INTO THE POSTERIOR VENTRAL TEGMENTAL AREA RESULTS IN A UNIQUE NEUROCHEMICAL RESPONSE WITHIN THE NUCLEUS ACCUMBENS SHELL
R.A. Waeliss, C.P. Knight, E.R. Brown, L.A. Pratt, E.A. Engleman, S.R. Hauser, Z.A. Rodd

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Ethanol (EtOH) and nicotine (NIC) interact in many ways to promote drug use. However, research predominantly focuses on a single drug of abuse. Recent studies indicate co-abuse of EtOH and NIC results in unique neuroadaptations compared to equivalent exposure to either drug alone. The posterior VTA (pVTA) is the primary location for dopamine (DA) neurons comprising the mesocorticolimbic pathway and are heterogeneous for a number of factors. One subset of VTA DA neurons have been shown to co-release glutamate. The current experiment examined the effects of rewarding (threshold) or subthreshold doses of EtOH, NIC, or EtOH+NIC microinjected into the posterior VTA on DA and glutamate release within the nucleus accumbens shell (AcbSh). In male Wistar rats, neurotransmitter levels were determined in the AcbSh following acute microinjections of artificial cerebrospinal fluid (aCSF), EtOH, NIC or EtOH+NIC. Animals received experimenter controlled microinjections into the pVTA designed to emulate intracranial self-administration levels (5 s injections, 15 s timeout period, 100 nL/injection over 10 min), concurrently with *in vivo* microdialysis of the AcbSh. Rats were microinjected with aCSF, subthreshold (100 mg%) and threshold (150 mg%) EtOH, subthreshold (10 μ M) or threshold (50 μ M) NIC, or combinations of subthreshold (100 mg% EtOH + 10 μ M NIC) or threshold (150 mg% EtOH + 50 μ M NIC) EtOH+NIC. DA levels within the AcbSh increased by 35% following microinjections of EtOH or NIC into the pVTA. EtOH+NIC resulted in an 80% increase of DA release which persisted over time compared to EtOH or NIC alone. Microinjection of EtOH or NIC alone had no effect on glutamate levels in the AcbSh. In contrast, co-administration of EtOH+NIC into the pVTA increased glutamate levels 40% in the AcbSh. These data indicate that an increase in glutamate release within the AcbSh was not dependent upon an increase in DA. Results suggest that only co-administration of EtOH+NIC activate pVTA DA/glutamate neurons projecting to the AcbSh. This specific activation could be part of the biological basis for previous work showing significant increases in BDNF levels within the AcbSh following co-administration of EtOH+NIC into the pVTA. The findings reveal that only EtOH+NIC co-administration/use results in distinct perturbations that could stimulate plasticity throughout the mesolimbic system and promote the development of addiction.

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