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Fear conditioning in mouse lines genetically selected for binge-like ethanol drinking

John C. Crabbe [a,b,*], Jason P. Schlumbohm [a,b], Wyatt Hack [a,b], Amanda M. Barkley-Levenson [a,b], Pamela Metten [a,b], K. Matthew Lattal [b]

[a] Portland Alcohol Research Center, VA Portland Health Care System, Portland, OR 97239, USA
[b] Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR 97239, USA

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ABSTRACT

The comorbidity of substance- and alcohol-use disorders (AUD) with other psychiatric conditions, especially those related to stress such as post-traumatic stress disorder (PTSD), is well-established. Binge-like intoxication is thought to be a crucial stage in the development of the chronic relapsing nature of the addictions, and self-medication through binge-like drinking is commonly seen in PTSD patients. We have selectively bred two separate High Drinking in the Dark (HDID-1 and HDID-2) mouse lines to reach high blood ethanol concentrations (BECs) after a 4-h period of access to 20% ethanol starting shortly after the onset of circadian dark. As an initial step toward the eventual goal of employing binge-prone HDID mice to study PTSD-like behavior including alcohol binge drinking, we sought first to determine their ability to acquire conditioned fear. We asked whether these mice acquired, generalized, or extinguished conditioned freezing to a greater or lesser extent than unselected control HS/Npt mice. In two experiments, we trained groups of 16 adult male mice in a standard conditioned fear protocol. Mice were tested for context-elicited freezing, and then, in a novel context, for cue-induced freezing. After extinction tests, renewal of conditioned fear was tested in the original context. Mice of all three genotypes showed typical fear responding. Context paired with shock elicited freezing behavior in a control experiment, but cue unpaired with shock did not. These studies indicate that fear learning *per se* does not appear to be influenced by genes causing predisposition to binge drinking, suggesting distinct neural mechanisms. However, HDID mice are shown to be a suitable model for studying the role of conditioned fear specifically in binge-like drinking.

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Introduction

Alcoholism and the alcohol-use disorder (AUD) spectrum afflict more than 18 million people in the USA aged 18 or older (Warren, 2009), and the annual cost to the US economy was estimated already to exceed $223 billion in 2009 (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011). Binge-like intoxication is thought to be a crucial stage in the development of the chronic relapsing nature of the addictions (Mandyam & Koob, 2012). Risk for AUDs is substantially heritable (Goldman, Oroszi, & Ducci, 2005). There are many rodent models for high levels of alcohol drinking. These models have generally been developed by selectively breeding for high preferential intake for 10% ethanol versus water when both fluids are offered continuously. Many such selected lines have been created and studied (for review, see Crabbe, 2014). One curious feature of these selected lines is that they generally do not drink in patterns that lead to behaviorally intoxicating blood ethanol concentrations (BECs). Unlike many humans diagnosed with alcohol dependence, they do not achieve the BEC (80 mg%) established by the NIAAA (2004) to define a binge.

Considering this to be a limitation of current models, we set out 10 years ago to develop a better model of focused, binge-like drinking in mice. Since rodents ingest most of their food and fluids early during their circadian dark period, we replaced the water bottle with a single bottle of 20% ethanol (a relatively high concentration for rodents) and found that C57BL/6J mice drank enough alcohol in a 4-h session of drinking in the dark (DID) to exceed 80 mg% BECs (Rhodes, Best, Belknap, Finn, & Crabbe, 2005).

* Corresponding author. Research (R&D 12), Portland VA Medical Center, 3710 SW US Veterans Hospital Road, Portland, OR 97239, USA. Tel.: +1 503 273 5298; fax: +1 503 273 5851.
E-mail address: crabbe@ohsu.edu (J.C. Crabbe).

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To develop a model enriched for genetic contributions to such drinking, we bred mice from a genetically heterogeneous population to produce a High Drinking in the Dark selected line (HDID-1). The basis for selection was BEC (not g/kg intake), and these animals showed increased BECs across generations and drank ethanol to the point of becoming behaviorally intoxicated (Crabbe et al., 2009). With continued selection, the current (32nd selected) generation of HDID-1 mice reach BECs averaging 180 mg%; they also have very high ethanol intake (6.9 g/kg in 4 h). A second, genetically independent replicate of this selection was initiated later. The HDID-2 (generation S26) reaches an average BEC of 155 mg% and drinks somewhat more ethanol than HDID-1. During the most recent test of the entire population to choose breeders, 58% of HDID-1 and 44% of HDID-2 mice reached BECs $\geq$160 mg%, twice the NIAAA standard for a binge. BECs $\geq$240 mg% (3 times the threshold) were reached in 25% of HDID-1 and 6% of HDID-2 mice. This is a unidirectional selection. Thus, no lines were bred for low BECs. Details of this selection's recent progress have been published (Crabbe et al., 2014).

A useful feature of selected lines is that if the lines differ (in this case, from their unselected control line, HS/Npt mice) on another trait, it is demonstrated that some of the genes leading to high DID-BEC also affect the correlated trait. Given the practical constraints of selection, this inference is greatly strengthened if one sees a parallel response in a genetically independent replicate of the selected line (Crabbe, Phillips, Kosobud, & Belknap, 1990).

The extensive comorbidity of AUD, other substance-use disorders, and other psychiatric disorders is well established (Kendler, Prescott, Myers, & Neale, 2003), notably including disorders clearly related to stress such as post-traumatic stress disorder (PTSD). Prevalence of PTSD among US military veterans is especially high (Davis, Bush, Kivlahan, Dobie, & Bradley, 2003) and was estimated to be between 6 and 24% depending on the definition of PTSD; comorbidity of AUD with either PTSD or depression was approximately 50% (Thomas et al., 2010). Currently, little is known about the biological mechanisms common to A/SUDs and PTSD (Norman et al., 2012). Combat experience leads young veterans to express a highly prevalent and disabling form of AUD, binge-like alcoholic drinking, leading to especially high BECs (Cucciare, Darrow, & Weingardt, 2011). The long-term goal of the studies we report here is to implement the HDID lines as a model of PTSD-like exacerbated binge drinking. Although the lines already achieve binge-like BECs and drink excessively, the BEC ranges reported above make it clear that these lines have not yet reached any biological limits to their intakes/BECs. Many rodent models of PTSD are based on classically conditioned fear, on the theory that PTSD resembles inappropriate generalization of a panic-like, anxious response to cues in the post-traumatic environment (Maren & Holmes, 2015). Thus, a predominant therapeutic approach is to decrease cue-induced fear responses by exposing patients to cues associated with trauma or anxiety, allowing the fearful response to diminish with repeated exposure through a process known as extinction. A common murine model for fear conditioning builds upon the unconditioned behavioral arrest (freezing) response displayed by mice to cues previously associated with foot shock (Fanselow, 1980; Kaouane et al., 2012). Robust freezing responses occur after a single pairing between a neutral conditioned stimulus (CS) and a biologically significant unconditioned stimulus (US). This learned response can persist across the lifespan of rodents (e.g., Gale et al., 2004) and, although it decreases over the course of repeated exposures to the CS in the absence of the US, conditioned freezing often returns even after successful extinction treatments (e.g., Lattal & Maughan, 2012).

As an initial step toward the eventual goal of employing our alcohol binge-prone HDID mice to study PTSD-related behavior, particularly including alcohol binge drinking, we sought first to determine their ability to acquire conditioned fear. We asked whether these mice acquired, generalized, or extinguished shock-induced freezing to a greater or lesser extent than unselected control HS/Npt mice. We used a protocol for assessing conditioned fear routinely used in the Lattal laboratory (Raybuck & Lattal, 2011). HDID-1 and HDID-2 mice have previously been shown to acquire a preference for a location associated with ethanol injections, and they do not differ from HS/Npt mice in that conditioned place preference (CPP) (Barkley-Levenson, Cunningham, Smitasin, & Crabbe, 2015). In contrast, when ethanol injections were used to establish a conditioned aversion to a novel taste (saline solution), both HDID-1 and HDID-2 mice showed reduced sensitivity to ethanol compared to HS/Npt. While a high dose of ethanol (4 g/kg, administered intraperitoneally [i.p.]) conditioned a strong aversion in all mice, a stronger conditioned taste aversion was seen in HS/Npt mice to an intermediate dose (2 g/kg) than in either HDID replicate line. All three genotypes showed equivalent taste conditioning induced by injections of lithium chloride (Barkley-Levenson et al., 2015). Although HDID mice do not seem to differ in ethanol reward sensitivity, they are less sensitive to the aversive properties of ethanol. If this is a general insensitivity to aversive outcomes, then one might expect that they would show deficits in fear conditioning. Alternatively, given that they show normal associative learning in associating reward with a context in a CPP procedure, they may show normal fear conditioning, which also involves associating a contextual stimulus with an outcome.

Experimental procedures

Animals and husbandry

Male mice from the HDID-1, HDID-2, and non-selected HS/Npt lines were bred in our colonies in the VA Portland Health Care System Veterinary Medical Unit. All mice were naïve at the beginning of each experiment and were between 70- and 135-days-old at the start of testing. HDID-1 mice were from the 29th selected generation and HDID-2 mice were from generations S22 and S23. HS/Npt mice were from filial generation G78. For the third experiment, HDID-1 mice of the 31st selection generation, HDID-2 mice of generation S25, and HS/Npt of filial generation G81 were used. The HS animals were the genetically heterogeneous population from which both HDID-1 and HDID-2 lines were selected, starting about 2 years apart. The HS/Npt animals were created by systematically intercrossing eight inbred mouse strains (Hitzemann, Dains, Kanes, & Hitzemann, 1994) and are maintained as 48 rotationally mated breeding pairs (Crabbe, Spence, Brown, & Metten, 2011).

Mice were maintained in standard plastic cages on Bed-o'Cobs® bedding (Andersons, Maumee, OH, USA) with stainless steel wire bar tops with a recess for chow. Rodent chow 5001 (PMI Nutrition International, Brentwood, MO, USA) and tap water were available *ad libitum*, and colonies and testing rooms were maintained on a 12-h:12-h reversed light:dark schedule (lights on at 9:30 PM, lights out at 9:30 AM) at a temperature of 21 $\pm$ 1 °C. Two weeks before the start of an experiment, mice were transferred to a procedure room with the same environmental conditions other than the light:dark schedule. During this time they acclimated to a forward light:dark schedule (lights on at 6:00 AM, lights off at 6:00 PM) in order to test mice in the light, as per standard protocols, during the human daytime. All procedures were approved by the VA Portland Health Care System Institutional Animal Care and Use Committee and were performed according to NIH Guidelines for the Care and Use of Laboratory Animals.