

The effect of doxycycline on alcohol consumption and sensitivity: consideration for inducible transgenic mouse models

Sally R McIver, Megan M Muccigrosso and Philip G Haydon

Department of Neuroscience, Tufts University, 136 Harrison Avenue, Boston, MA 02111, USA

Corresponding author: Sally R McIver. Email: Sally.McIver@tufts.edu

Abstract

Neuroinflammation is known to elicit numerous changes in brain physiology and is associated with various pathologies, including neurodegenerative diseases, and behaviors, such as sleep and acute illness. In addition, there is accumulating evidence that the behavioral response to alcohol is affected by perturbations to the neuroimmune system. Recent studies have shown that administration of proinflammatory mediators increases alcohol consumption, while anti-inflammatory drugs, such as minocycline, decrease consumption. Doxycycline is an anti-inflammatory mediator and a tetracycline derivative, and is commonly used in the tetracycline regulatory system, a transgenic approach widely accredited for its inducible and reversible nature. Given the established link between anti-inflammatory agents and response to and consumption of alcohol, and because the tetracycline regulatory system is becoming increasingly employed for genetic manipulations and behavioral phenotyping, we investigated the effect of doxycycline administration on alcohol sensitivity and consumption. Two independent transgenic lines containing a tetracycline transactivator transgene or the tetracycline operator promoter insertion, along with wild-type littermate mice (C57Bl/6J), were used to measure changes in alcohol consumption, alcohol-induced motor impairment and sedation, and blood alcohol concentration with doxycycline administration (40 mg/kg in chow). Using repeated sessions of the drinking-in-the-dark paradigm, we found that doxycycline consistently reduced consumption of 20% alcohol during two- and four-hour access. Doxycycline also increased sensitivity to the motor-impairing effects of alcohol (2 g/kg), and the duration of loss of righting reflex after ethanol injection (3.5 g/kg), without causing a significant alteration in blood alcohol levels. Despite the many advantages of using a tetracycline-regulated transgenic approach, it is important to consider the effects of doxycycline administration in behaviors that may be influenced by neuroinflammation, including alcohol behaviors.

Keywords: doxycycline, alcohol, tetracycline, transgenic

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Introduction

There are numerous genetic and environmental variables that impact the behavioral response to alcohol, making it especially difficult to clinically predict risk for alcohol misuse or dependency. These variables, in combination with the fact that alcohol has widespread targets throughout the central nervous system, have impeded progress on understanding the cellular and molecular pathways involved in acute and chronic alcohol use behaviors. Similarly, there are limitations to pharmacological approaches for examining alcohol behaviors, since alcohol is known to modulate virtually every neurotransmitter system, but may do so differentially among different cell types and brain regions. The development of transgenic mouse lines allowing for inducible, region- and/or cell-specific transgene expression has offered a new tool for

investigating more selective targets of alcohol.¹ In particular, these mice are created by crossing two lines of transgenic mice – one line expresses a cell-specific promoter driving expression of the tetracycline transactivator (tTA), and the other line uses the tetracycline operator (tetO) to drive expression of a transgene of interest; transgene expression can then be switched on and off in bigenic mice via oral administration of the tetracycline derivative, doxycycline (dox).² This transgenic approach theoretically also has the benefit of within-animal comparisons (on versus off dox), further addressing the intrinsic and well-documented variability of animal behaviors. Dox is a second-generation tetracycline that exhibits high lipid solubility, relative to first-generation tetracyclines, allowing for higher absorption and widespread tissue distribution.³ The capacity for therapeutic relevance of dox and its

related tetracycline derivative, minocycline, is ever-increasing, especially given recent studies showing that these antibiotics effectively modulate neuroinflammation. For example, they have been shown to reduce proinflammatory cytokine production and subsequent ischemic injury in experimental models of hypoxia-ischemia and focal cerebral ischemia.^{4,5} Similarly, a side-by-side comparison of the effect of doxycycline and minocycline on cell cultures exposed to hypoxia indicate that these tetracyclines attenuate microglia activation and hypoxic neuronal death with equal efficiency.⁶ Furthermore, dox has been shown to effectively reduce neuroinflammation and cell death in an experimental model of Parkinson's disease.⁷

Recent studies have begun to address the impact of neuroinflammatory modulators on alcohol behaviors. For example, minocycline was shown to reduce alcohol consumption and to differentially alter sensitivity to the motor-impairing and sedative effects of alcohol, whereas the proinflammatory agent, lipopolysaccharide (LPS), was shown to increase consumption.⁸⁻¹⁰ Given these recent studies describing the impact of neuroinflammatory modulators on alcohol consumption, and the increasing employment of the tetracycline regulatory system for transgene expression, the effect of dox on response to alcohol should be taken into consideration. In this study, the effect of dox on alcohol consumption, sensitivity to alcohol, and blood alcohol metabolism, was tested in either wild-type mice or mice expressing either the tetracycline transactivator or the tetracycline operator.

Materials and methods

Transgenic mouse lines

Three genotypes of mice were used for experiments, all of which were raised on the C57Bl/6J background. In one line of transgenic mice, the astrocyte-specific promoter, glial fibrillary acidic protein, was used to drive expression of the tetracycline transactivator ('tTA+ mice'); in the second line, the tetracycline operator was used to drive expression of two transgenes, including the reporter protein enhanced green fluorescent protein (eGFP) ('tetO+ mice'); the third group of mice were wild-type littermate controls ('WT mice'). The generation of these lines of mice, as well as the bigenic crossing of the tTA+ and tetO+ mice, have been previously described in other studies.¹¹⁻¹³ However, it should be noted that since bigenic genotypes (tTA+/tetO+) are required for functional transgene expression, none of the mice used in the current study expressed the functional transgenes described in the aforementioned studies. Inherent eGFP expression was examined in postmortem brain tissue using epifluorescent microscopy to confirm an absence of transgene expression. Dox was administered orally via consumption (*ad lib*) of standard mouse chow containing 40 mg dox per 1 kg food (Bio-Serv, Frenchtown, NJ, USA).

All dams were placed on a dox diet (*ad lib*) throughout gestation and during postnatal rearing, allowing pups to be maintained on dox (40 mg/kg) throughout development. Upon weaning (4 weeks old), mice were given regular

animal chow until further testing (>8 weeks old). This regimen is of standard use for experiments using inducible transgenic mice and is employed to avoid non-specific or compensatory effects that may otherwise result from genetic modifications occurring during development (a limitation which is more likely to occur with constitutive transgenic expression).¹¹⁻¹³

Dox (40 mg/kg) or regular chow was readministered for two to three weeks prior to experimental testing and mice were allowed to consume *ad lib* throughout all experimental procedures. Mice were housed in a 12/12 h light/dark cycle unless otherwise specified.

Behavioral tests

To test sensitivity to the motor-impairing effects of alcohol, mice were trained to walk on the rotarod at a speed of 22 rpm for 60 s, one day prior to testing. On the day of testing, a baseline performance of 60 s on the rotarod was confirmed prior to administration of an intoxicating dose of ethanol (2.0 g/kg; intraperitoneally). The latency to fall off the rotarod was measured 15, 30, 45, 60 and 80 min post-injection, and plotted as percent of baseline ('performance'). Loss of righting reflex (LORR) was induced using an intraperitoneal injection of 3.5 g/kg ethanol and the duration of LORR was measured as an indicator of sensitivity to the sedative effects of alcohol. Approximately five minutes postinjection, mice were placed on their backs in a V-shaped container (~90° angle), and the LORR duration was marked by the amount of time required for the mouse to become upright (at least three paws grounded) three times in one minute. Motor performance and LORR following alcohol injection were measured during the light cycle, approximately seven hours after lights on.

Mice were housed in a 12/12 h reverse light/dark cycle for the drinking-in-the-dark (DID) experiments. DID, a model of binge drinking that has been shown to produce high consumption levels in C57Bl/6J mice, was carried out according to Rhodes *et al.*¹⁴ Briefly, beginning three hours into the onset of the dark cycle (subjective daytime), the water bottles were removed and replaced with 20% ethanol. Mice were given two hours to freely consume the alcohol on days 1-3, and on day 4, mice were given four hours access to alcohol. Alcohol bottles were measured before and after consumption, mice were weighed at the end of each DID session, and total consumption was plotted as g/kg and averaged for days 2-3, and day 4. Mice were tested twice for each experiment (on or off dox).

Blood alcohol measurements

Blood samples were taken from mice using submandibular puncture and immediately placed on ice at the end of every experiment as follows: approximately 90 min after injection for the rotarod tests; approximately 2 h for the LORR tests (after all mice resumed the righting reflex); and immediately following the end of the four hours access to 20% alcohol (day 4 of DID). Serum was collected and blood alcohol content was measured using the Ethanol Assay kit

(Sekisui/Genzyme Diagnostics, Framingham, MA, USA) according to the manufacturer's instructions.

Statistics

All mice were tested on and off dox using a counter-balanced design, and the data from all the three genotypes (tTA+, tetO+ and WT) were pooled, collectively representing 'littermate controls' typically used for comparison with tet-regulatory inducible transgenic mice (tTA+/tetO+). Repeated measures analysis of variance (ANOVA) with pairwise comparisons was used to examine the effect of dox on LORR duration ($n = 9$) and to compare blood alcohol content on and off dox for all three tests described. Repeated measures two-way ANOVA with pairwise comparisons was used to examine the effect of dox on alcohol-induced motor impairment on the rotarod ($n = 17$) and for alcohol consumption in the DID test ($n = 9$). SigmaPlot software was used for all data analyses (Systat Software, Inc., San Jose, CA, USA).

Results

Doxycycline administration reduces alcohol consumption

Using the DID paradigm, alcohol consumption was measured in mice on and off a dox diet (*ad lib*) using a counter-balanced design. As shown in Figure 1a, mice consumed significantly less 20% alcohol during two hours and four hours access when on dox (two-way ANOVA; main effect for dox treatment; $P < 0.05$). Though the mice on dox tended to have higher blood alcohol levels, the trend was not significant (Figure 1b; $P = 0.08$, one-way ANOVA).

Doxycycline increases sensitivity to the motor-impairing and sedative effects of alcohol

To examine sensitivity to the motor-impairing effects of alcohol, ability to stay on the rotarod was measured following administration of 2.0 g/kg ethanol (intraperitoneally). Mice were significantly more impaired on the rotarod when tested on dox (Figure 1c; two-way ANOVA; main effect for treatment (dox), $P < 0.001$ and time, $P < 0.001$; *post hoc* Bonferroni's *t*-test $P < 0.05$ at 30, 45, 60 and 80 min). However, there was no effect on blood alcohol levels (Figure 1d; one-way ANOVA; $P = 0.28$). Similarly, dox significantly increased LORR duration in response to 3.5 g/kg (intraperitoneally; Figure 1e; one-way ANOVA; $P < 0.05$), without having a significant impact on blood alcohol levels (Figure 1f; one-way ANOVA; $P = 0.30$). These results show that dox increases behavioral sensitivity to alcohol without altering blood alcohol concentration, when alcohol is administered systemically.

Discussion

Alcohol is known to alter cytokine production in various tissues, including the brain, to the extent that measurements of circulating cytokines in cerebrospinal fluid may offer potential as a diagnostic tool for alcoholism.^{15,16} The

bidirectional impact of neuroinflammatory modulators on alcohol behaviors has recently been of great interest. For example, treatment with the proinflammatory and bacterial endotoxin, LPS, increases alcohol consumption, whereas the anti-inflammatory and tetracycline derivative, minocycline, decreases alcohol consumption and differentially impacts alcohol sensitivity in mice.^{8–10} Our results showing reduced consumption of and increased sensitivity to alcohol during treatment with another tetracycline derivative, dox, are consistent with these findings. We also extended on previous findings by showing that dox does not alter blood alcohol clearance *per se*, suggesting that dox acts centrally to impact alcohol sensitivity.

Here, we show that oral dox intake increases sensitivity to the motor-impairing effect of alcohol, which is consistent with a previous study showing similar results with systemic minocycline administration; however, our results showing increased alcohol-induced sedation with dox treatment are in contrast with those reported following minocycline treatment.⁹ It is not entirely clear why minocycline treatment has differential effects on alcohol-induced sedation, relative to dox treatment. One possibility is that there are subtle differences in downstream mediators of these two drugs. For example, though chemically similar, dox has been shown to be a more potent inhibitor of matrix metalloproteinases (MMPs).¹⁷ MMP2 has been found to be increased in the kidneys of rats following long-term alcohol consumption, but whether or not MMPs contribute to the acute behavioral response to alcohol (e.g. sedation) is not known.¹⁸ It is also likely that the discrepancy in the sedative response to alcohol is due to differences in the routes and duration of drug administration. Specifically, minocycline treatment was administered systemically (intraperitoneally) daily for three days prior to alcohol exposure; whereas in the current study, dox was administered orally and continuously for at least two weeks prior to testing alcohol sensitivity. It is possible that the absorption and tissue distribution of these drugs vary depending on the route and duration of administration, consequently impacting sensitivity to the sedative effects of alcohol.

Collectively, these results highlight a potential confound in using tetracycline-regulated transgene expression systems in experimental models for application to alcohol studies and also lend support to pre-existing evidence showing the effect of neuroinflammatory agents on alcohol behaviors.

Author contributions: SRM and MMM designed the experiments and carried out data analysis. SRM drafted the manuscript, and PGH contributed to the draft of the manuscript. All authors contributed to the interpretation of results and read and approved the final manuscript.

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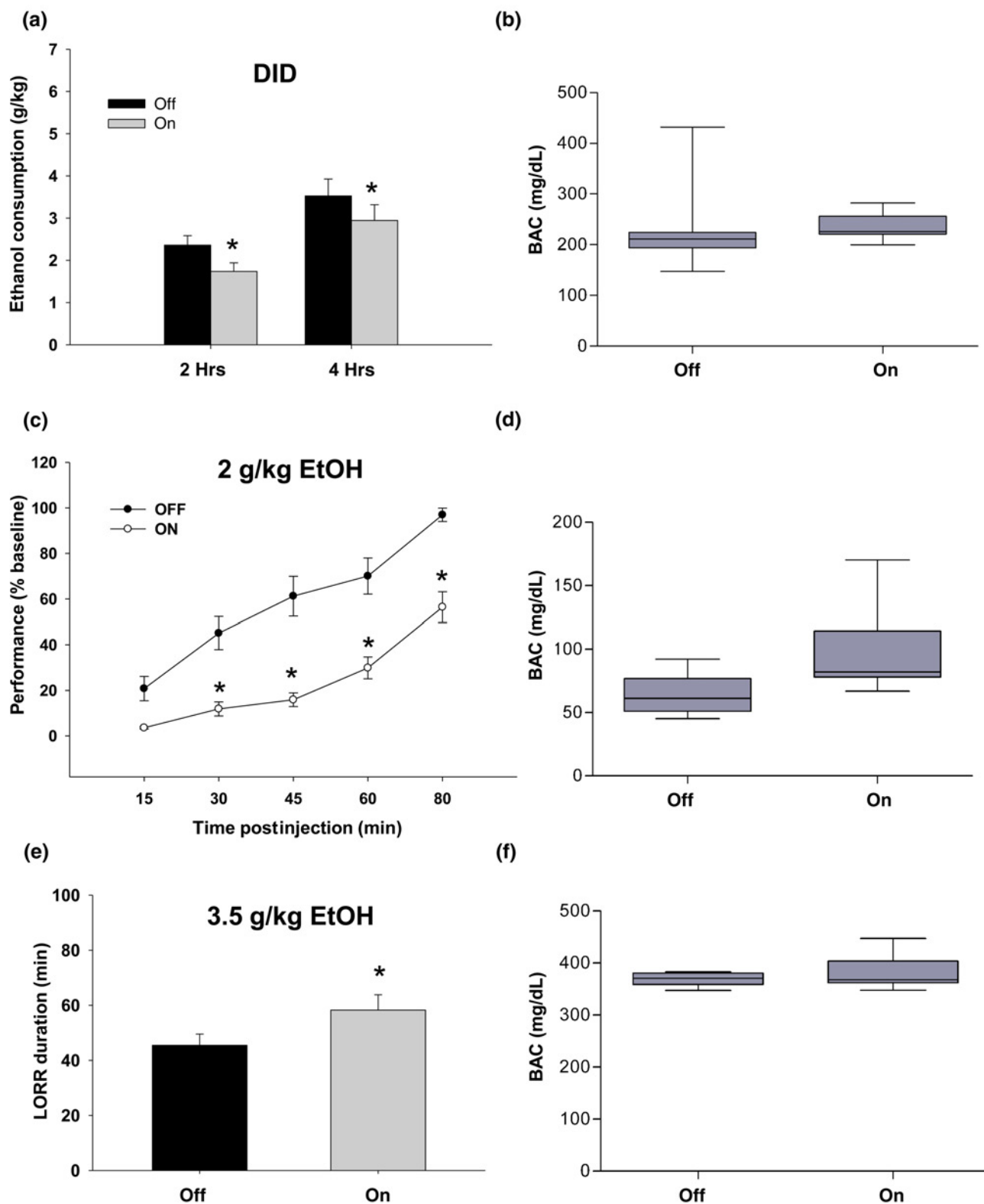


Figure 1 Doxycycline administration decreases ethanol consumption, increases sensitivity to the motor-impairing effects of alcohol, and alters blood alcohol levels. (a) Mice on dox consume significantly less when given two hours or four hours access to 20% alcohol in the drinking-in-the-dark (DID) paradigm ($n = 9$; $*P < 0.05$; main effect for dox treatment, repeated measures two-way analysis of variance). Because DID consumption was measured during four independent cycles (2 on dox, 2 off dox; counterbalanced), the effect of order of dox administration on DID consumption was also examined, and proved not to be significant (main effect for order of dox treatment; $P > 0.05$). Likewise, there was no significant interaction between dox treatment and order of dox administration on alcohol consumption ($P > 0.05$). (b) There was no significant effect of dox administration on blood alcohol levels when measured after four hours DID ($n = 9$; $P = 0.08$). (c) Dox administration significantly increases sensitivity to the motor-impairing effects of alcohol, marked by reduced performance on the rotarod following 2 g/kg alcohol ($n = 17$; $*P < 0.001$). (d) Dox does not affect blood alcohol levels in mice when measured two hours after the injection ($n = 17$; $P = 0.28$). (e) Dox also significantly increases sensitivity to the sedative effects of alcohol, as mice on dox have increased duration of loss of righting reflex (LORR) following injection of 3.5 g/kg alcohol ($n = 9$; $*P < 0.05$). (f) Interestingly, there was no significant difference in blood alcohol content measured 90 min after injection of 3.5 g/kg alcohol ($n = 9$; $P = 0.30$). For (b), (d) and (f), horizontal bars in box plots represent median values; vertical bars represent SEM

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