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Article type : Original Research Article

Brucine N-oxide reduces ethanol intake and preference in alcohol-preferring male Fawn-Hooded rats

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Abstract

Background: Alcohol use disorder places a heavy burden on global public health systems and thus is in urgent need of improved pharmacotherapies. Previously, our group has demonstrated that 30 mg/kg of the indole alkaloid brucine significantly attenuates alcohol drinking behavior; however, the high toxicity, poor water solubility, short half-life, and limited therapeutic window of brucine restrain its clinical application as an anti-alcoholism medication. We subsequently hypothesized that the oxide of brucine (brucine N-oxide) would produce a similar behavioral effect without the risk profile associated with brucine.

Methods: Male Fawn-Hooded rats with high innate alcohol preference underwent two-bottle choice procedures (Experiments 1-3). Experiment 1 examined the effects of 7 daily BNO injections of 0, 30, 50 or 70 mg/kg (s.c.) on voluntary alcohol consumption (n = 9/group). Experiment 2 evaluated the impact of a single dose of 0 or 70 mg/kg BNO on the

increased alcohol intake induced by a 4d alcohol deprivation (n = 8/group). Experiment 3 tested the effect of 7 daily BNO injections of 0 or 70 mg/kg (s.c.) on sucrose preference (n = 6/group). Experiment 4 measured the median lethal dose (LD50) values of BNO and brucine to compare their acute toxicity in rats. Experiment 5 tested whether BNO (0, 30, 50 and 70 mg/kg, s.c.) affected locomotor activity using an open-field paradigm (n= 8/group). Finally, Experiment 6 evaluated the possible conditioned rewarding effects of 0, 30, 50, and 70 mg/kg BNO using the conditioned place preference paradigm (n = 6/group).

Results: BNO administration dose-dependently attenuated alcohol consumption without affecting food intake, total fluid consumption or the natural preference for a sucrose solution, with 70 mg/kg BNO reducing consumption by 22.8%. A single dose of 70 mg/kg BNO significantly inhibited the alcohol deprivation effect. The LD50 values of BNO and brucine in rats were determined to be 1103.5 ± 177.0 mg/kg and 264.6 ± 17.7 mg/kg, respectively. Finally, BNO administration did not affect spontaneous locomotor activity or induce a place preference.

Conclusions: BNO may help to control excessive alcohol use and should be considered a treatment strategy for future study and development.

Keywords : brucine N-oxide; alcohol; alcohol use disorder; Fawn-Hooded (FH/Wjd) rat; glycine receptor

1. Introduction

People drink alcohol to socialize, relax and relieve pressure in contemporary society. However, some individuals can develop alcohol use disorders (AUD) after repeated alcohol consumption, which induces chronic and progressive alterations in the central nervous system (CNS). AUD inflicts economic and social losses on the individual as well as society at large and urgently requires the development of more effective therapies. A global survey on alcohol and health has revealed that harmful alcohol use results in 3,000,000 deaths and contributes to 5% of the burden of diseases worldwide annually (World Health Organization, 2018). Moreover, excessive alcohol consumption positively correlates with the development of liver, oesophagus, larynx, pharynx, oral cavity and female breast cancers (Bagnardi et al., 2001, 2013; Talamini et al., 2002; Znaor et al., 2003; Turati et al., 2013).

Clinically, naltrexone and acamprosate are prescribed to control the symptoms of AUD; however, the side effects of naltrexone and the low bioavailability of acamprosate may limit their therapeutic usage (Kaaber et al., 1987; Croop et al., 1997; Jupp & Lawrence, 2010; Witkiewitz et al., 2012). Consequently, it is necessary to develop medications with higher efficacy and fewer side effects for better compliance from patients.

Semen strychni is a traditional Chinese medicine that has been used for hundreds of years due to its significant analgesic and anti-inflammatory properties (Yin et al., 2003). The major active components of this compound are indole alkaloids, including brucine (Fig. 1a) and brucine N-oxide (BNO; Fig. 1b). Previously, we found that brucine had an inhibitory effect on alcohol drinking behavior in FH/Wjd rats, an innate alcohol-preferring rat strain (Li et al., 2014). However, brucine is classified as a toxic substance with a high elimination rate and a narrow therapeutic window, which impedes its potential translation to clinical settings (Yin et al., 2003; Li et al., 2014). Because oxidation is a common pathway of detoxification and delay of the elimination of alkaloids (Ziegler & Gold, 1971; Rose & Castagnoli, 1983), we hypothesized that the use of BNO, an oxide of brucine, might result in a similar anti-alcohol-drinking effect, but with negligible toxicity and a wide therapeutic dose range.

In this study, we first investigated the effects of BNO administration on alcohol drinking phenotypes and sucrose preference using an oral drinking paradigm in rats. We further investigated the acute toxicity of brucine and BNO. Finally, we determined whether BNO alters locomotor activity or induces a place conditioning effect.

2. Materials and methods

2.1. Animals

FH/Wjd rats were generously provided by Prof. Andrew J. Lawrence at the Florey Institute of Neuroscience and Mental Health, University of Melbourne (Melbourne, Australia) and bred at the Department of Laboratory Animal Sciences, Peking University Health Science Center (License number: SCXK-(Jing) 2011-0012). Experimental rats were housed and raised in hyaline plastic boxes and had free access to water and food in a condition-controlled room (Relative humidity: 50% ± 10%; temperature: 22 °C ± 1 °C) on a 12-h light/darkness cycle (on 8:00 am, off 8:00 pm). We used male rats of approximately 10 weeks old and habituated them to the environment prior to experiments. The experimental procedures were approved by the Local Committee on Animal Care and Use. Animals were treated according to the NIH Guide for the Care and Use of Laboratory Animals.

133

134 2.2. Drugs

135 BNO hydrate (Sigma-Aldrich, St. Louis, MO, USA) and morphine
136 hydrochloride (Qinghai Pharmaceutical Plant, Qinghai, China) were
137 prepared with saline and administered subcutaneously in a volume of 1
138 mL/kg. Ethanol (5%, 10%, v/v; Beijing Chemical Factory, Beijing, China)
139 and sucrose (0.5%, w/v; Beijing Chemical Factory, Beijing, China)
140 solutions were prepared in tap water.

141

142 2.3. Ethanol two-bottle choice drinking test

143 This test was performed according to the classic paradigm (Pandey et al.,
144 2004; Li et al., 2014; Eisenhardt et al., 2015). FH/Wjd rats were housed
145 separately in the home cage and given one bottle containing 5% ethanol
146 solution and one water bottle continuously for 8 consecutive weeks as an
147 adaptive phase to ensure a stable level of ethanol consumption prior to
148 testing. A 5% solution was used because FH/Wjd rats have a higher
149 preference for ethanol at this concentration (Li et al., 2014). Solutions
150 were replaced and the positions of the bottles were switched daily.
151 Thirty-six rats with high alcohol preference (> 65%) were divided into 4
152 groups using a randomized block design (n = 9/group). BNO treatment at
153 0 mg/kg (saline), 30, 50 and 70 mg/kg was injected twice a day (at 8:00
154 am and 7:30 pm) for 7d. The procedure lasted for 13 days, which

included a 3d baseline period, 7d of drug treatment and a 3d post-treatment response measurement. Ethanol, water and food consumption were monitored every 24 h.

2.4. Alcohol deprivation test

The alcohol deprivation effect is characterized by a pronounced but transient elevation of ethanol consumption after a period of abstinence in rodents, which model alcohol relapse phenotypes (Spanagel et al., 1996; McBride et al., 2002; Rezvani et al., 2002; Li et al., 2014; Vengeliene et al., 2014). During this procedure, 24 alcohol-preferring FH/Wjd rats were given 10% ethanol and water for 8 weeks. The concentration of 10% was used based on higher daily alcohol consumption in FH/Wjd rats (Li et al., 2014). Rats were randomly divided into 3 groups: one baseline and two groups given a 4d alcohol deprivation (n = 8/group). At 7:30 pm of the last day, the deprivation groups were injected with 0 or 70 mg/kg BNO. The ethanol bottle was returned, and ethanol and water intake were recorded at 24 and 48 h.

2.5. Sucrose two-bottle choice drinking test

Here, a two-bottle choice paradigm was employed to assess whether BNO treatment affects the natural preference for a sucrose solution (Hu et al., 2011). FH/Wjd rats were provided with one bottle containing 0.5%

177 sucrose and one water bottle. Drinking solutions were replaced and the
178 positions of the bottles were switched daily. Twelve rats (sucrose
179 preference > 60%) were divided into 2 groups (n = 6/group) and
180 administered 0 or 70 mg/kg twice daily (at 8:00 am and 7:30 pm), with
181 sucrose preference monitored every 24 h.
182
183 2.6. Acute toxicity test
184 In this test, a modified Up-and-Down procedure was adopted to
185 determine the acute toxicity of BNO and brucine (Meyer et al., 2005; Xu,
186 1991). Here, 20 FH/Wjd rats (10 male and 10 female) for each alkaloid
187 were used to measure median lethal dose (LD50) values. Rats were fasted
188 overnight prior to BNO or brucine administration. Rats remaining alive
189 for 48 h after a single dose of BNO or brucine were classified using the
190 term “survival”; otherwise, a classification of “death” was given. LD50
191 values were calculated based on doses, numbers of survivals and deaths.
192
193 2.7. Locomotor activity test
194 An open-field test was employed to assess the effect of BNO on general
195 locomotor activity (Wen et al., 2012; Li et al., 2014). Thirty-two FH/Wjd
196 rats were separated into 4 groups (n = 8/group). Following injections with
197 0, 30 , 50 or 70 mg/kg BNO, rodents were placed into 4 identical
198 sound-attenuating chambers (49 cm × 49 cm × 54 cm, without ceiling) to

measure locomotor activity for 4 h. Total horizontal distance was monitored and recorded with DigBehv spontaneous activity monitors, and then analyzed using DigBehv software Version 2.0 (Shanghai Jiliang Software Technology Co. Ltd., China). Locomotion was calculated as horizontal traveling distance in 10min bins.

2.8. Conditioned Place Preference (CPP)

CPP measures the ability of a previously novel environment to acquire conditioned reinforcing properties when paired with a putatively rewarding or aversive stimulus, such as a drug, via Pavlovian learning (Childs et al., 2019). Conditioned reward using CPP is oftentimes assessed by the difference in time spent between a drug-paired context and a non-drug context. Here, CPP procedures were utilized to evaluate the conditioned reinforcing profile of BNO. We used an unbiased device comprised of 3 different chambers (a middle chamber of 14 cm×23 cm×20 cm, L×W×H; 2 side chambers of 28 cm×23 cm×20 cm, L×W×H) separated by a retractable guillotine door (Liu et al., 2012; Zhang et al., 2012). The side chambers were defined as distinguishable conditioning rooms with 2 somatosensory cues (visual: 5 radially-arranged or 4 squarely-arranged low-power light bulbs; tactile: stainless steel rod or stainless steel mesh floor). Behavior was monitored by 3 infrared photocells (3 cm above the floor) in each chamber. One day prior to

221 experiments, rats were placed in the chambers to habituate. Our
222 procedure then proceeded as follows over 10 consecutive days: a 15-min
223 preconditioning session (day 1; drug free), eight 45-min conditioning
224 sessions (day 2 to day 9; drug or saline treatment), and a 15-min test
225 session (day 10; drug free). On day 1, rats were placed into the central
226 compartment of the apparatus without the guillotine doors. The time
227 spent in each compartment was determined to measure their natural place
228 preference (exclusion criteria: time difference >120 s between the period
229 in two side compartments). In total, 30 rats met the inclusion criteria and
230 were separated into 5 groups (n = 6/group). On conditioning days, each
231 rat was trained with alternative injections of saline (unpaired
232 compartment) or drugs (0, 30, 50, and 70 mg/kg BNO or 6 mg/kg
233 morphine; paired compartment) and then immediately placed into the
234 proper compartments. Morphine at 6 mg/kg was used as a positive control
235 to induce a CPP response (Zhang et al., 2012). On day 10, the rats were
236 given access to the entire apparatus, and the ratio of time spent (s) in the
237 drug-paired side to that in the saline-paired side was calculated as the
238 CPP score.
239
240 2.8. Statistical Analysis
241 Data from two-bottle choice drinking paradigms and locomotor activity
242 test were analyzed using repeated measures analysis of variance

(RM-ANOVA) (factors: treatment, day), followed by LSD post-hoc tests. For ADE and CPP tests, unpaired t tests and one-way ANOVA, respectively, were used to analyze the data. Data were expressed as the mean ± SEM. The level of significance was $p < 0.05$.

3. Results

3.1. BNO treatment reduce ethanol intake and preference

The ethanol two-bottle choice paradigm has been extensively employed to model human alcohol drinking (Rezvani et al., 2002). BNO treatment led to a significant reduction in ethanol intake [$F(3, 32) = 5.88$, $p < 0.01$] (Fig. 2A) and preference [$F(3, 32) = 3.57$, $p < 0.05$] (Fig. 2B) during the drug-treatment period. BNO injections at 70 mg/kg significantly inhibited daily voluntary alcohol intake and preference by 22.8% and 14.2%, respectively, during the 7d treatment period. The inhibitory effect of BNO on cumulative ethanol intake was also dose-dependent, as shown in Fig. 3 (50 mg/kg: $p < 0.05$; 70 mg/kg: $p < 0.001$). Total fluid consumption was unchanged [$F(3, 32) = 2.58$, not significant (NS)], while water intake showed a clear dose-dependent trend toward an increase, but no overall statistical significance [$F(3, 32) = 2.78$, NS] (Fig. 2C). Food intake [$F(3, 32) = 2.67$, NS] (Fig. 2D) did not differ between groups. Notably, there was a sustained inhibition of alcohol intake, which remained below the baseline for at least 3 days post BNO administration.

3.2. BNO treatment inhibits an alcohol deprivation effect

A marked enhancement in ethanol intake in alcohol-preferring animals after a period of abstinence is referred to as an alcohol deprivation effect (ADE) (Rezvani et al., 2002; Li et al., 2014). Here, we monitored the drinking behavior in rats after ethanol was reintroduced. We found that acute 70 mg/kg BNO administration inhibited ethanol consumption (Fig. 4A; d1: $t(14) = 5.388$, $p < 0.01$; d2: $t(14) = 3.743$, $p < 0.01$) and preference (Fig. 4B; d1: $t(14) = 6.775$, $p < 0.01$; d2: $t(14) = 2.328$, $p < 0.05$) in alcohol-preferring FH/Wjd rats, without altering total fluid intake (Fig. 4C; d1: $t(14) = 1.461$, NS; d2: $t(14) = 2.061$, NS), as compared to the saline-treated group. In particular, ethanol consumption in the BNO-treatment group was inhibited by 44.4% (7.17 $\pm$ 0.5 g/kg vs 4.0 $\pm$ 0.38 g/kg), while ethanol preference was attenuated by 34.1% (92.1% $\pm$ 2.2% vs 58% $\pm$ 4.9%), on the first ADE test (d1). To draw a robust conclusion, the effectiveness of this paradigm was confirmed by the comparisons in ethanol intake (Fig. 4A; d1: $t(14) = -2.548$, $p < 0.05$; d2: $t(14) = -2.97$, $p < 0.05$) and preference (Fig. 4B; d1: $t(14) = -4.243$, $p<0.01$; d2: $t(14) = -3.922$, $p < 0.01$) between the baseline and saline-treated groups.

3.3. BNO treatment does not alter preference for a natural reward

Here, we examined whether the effect of BNO treatment on alcohol drinking is specific or extends to natural rewards. BNO treatment did not result in an alteration of sucrose preference ($F(1, 10) = 0.0004$, NS) (Fig. 5), suggesting that the BNO effect on alcohol drinking is alcohol-specific and not a non-specific effect on rewarding stimuli.

3.4 BNO has lower acute toxicity than brucine

Using a modified Up-and-Down procedure, we measured the LD50 value of BNO in rats as 1103.5 ± 177.0 mg/kg, which is lower than that of brucine at 264.6±17.7 mg/kg. These data indicate that BNO has a higher safety profile than brucine, and thus is more appropriate for future study and potential clinical development.

3.5. BNO treatment does not influence locomotor activity

There was no statistical difference among the groups in horizontal locomotor activity either in 10-min bins analyzed using a two-way RM-ANOVA [$F(3, 28) = 0.796$, NS] (Fig. 6) or across the 4 h test using a one-way ANOVA [$F(3, 28) = 0.796$, NS] (Fig.6 inset). These results show that general locomotor activity is not altered by BNO treatment at behaviorally-effective doses.

3.6. BNO does not induce side preference or aversion

Saline- and BNO-treated rats did not differ in CPP scores [F(3, 20) =1.401, NS] (Fig.7). In contrast, as expected, rats administered 6 mg/kg morphine demonstrated a significant CPP ($t(10) = -2.8$, $p < 0.05$), confirming the effectiveness of this paradigm (Zhang et al., 2012). BNO treatment at 30, 50 and 70 mg/kg did not produce a place preference or aversion, suggesting that it has no conditioned rewarding or aversive properties.

4. Discussion

Harmful alcohol use has become a public health issue that urgently requires effective and safe interventions. In this study, we demonstrated that treatment with BNO exerted a significant inhibitory effect on ethanol intake and preference in alcohol-preferring FH/Wjd rats in a dose-dependent fashion. BNO injections at 70 mg/kg (twice daily for 7d) decreased daily ethanol consumption by 22.8% (3.90 ± 0.56 g/kg versus 3.01 ± 0.91 g/kg) and preference by 14.2% (91.65% ± 9.51% versus 77.45% ± 21.84%) during the drug-treatment period. The current data also indicate that BNO resulted in a specific amelioration of excessive drinking phenotypes, as repeated 70 mg/kg BNO treatment did not impair the natural preference of FH/Wjd rats for sucrose solution. Furthermore, both food and total fluid intake were unaffected by BNO. Importantly, we also did not observe the development of a tolerance effect to repeated

BNO injections during the 7d treatment period. This is in contrast to acamprosate, which can result in tolerance if administered repeatedly (Lidö et al., 2012). Our data preliminarily suggest that BNO can be developed into an effective medication with high specificity and no known tolerance.

The ADE is characterized by a pronounced but short-term increase in ethanol consumption in laboratory animals, which is used to mimic human relapse-like phenotypes with reliable face and predictive validity (Spanagel et al., 1996; McBride et al., 2002; Rezvani et al., 2002). The amplitude of ADE is highly correlated with the alcohol sensitivity of animals, determined by genetic expression (Vengeliene et al., 2014). Vengeliene et al proposed that rats rather than mice are a more appropriate system to mimic alcohol relapse drinking and examine the relapse-relieving properties of candidate compounds, finding that rats showed stable ADEs after repeated alcohol deprivation, predictable compulsive drinking phenotypes after long-term alcohol intoxication, and reliable, not paradoxical, pharmacological effects after certain interventions compared to mice (Vengeliene et al., 2014). Therefore, FH/Wjd rats with high alcohol preference provide an appropriate model to screen the therapeutic compounds for AUD treatment, and to clarify the underlying neurobiology of relapse. In this study, alcoholic FH/Wjd

rats had 8-week alcohol access and stable intake prior to alcohol deprivation. We demonstrated that a single 70 mg/kg BNO administration resulted in a significant inhibition of ethanol relapse phenotypes (consumption by 44.4% and preference by 34.1%) on d1 of re-exposure. These data demonstrate that BNO may have anti-relapse effects, which is critical in the development of pharmacotherapies for AUDs.

Based on our findings, we predict that BNO medications may have low toxicity and few side effects. We determined that the LD50 of BNO was significantly less than that of brucine, measured using a modified Up-and-Down procedure (Xu, et al., 1991; Meyer et al., 2005). Comparatively, the effective doses are approximately 70 mg/kg and 30 mg/kg, for BNO and brucine, respectively, in alcohol-preferring FH/Wjd rats. Thus, BNO has much lower toxicity and a wider therapeutic window compared to brucine (Chen et al., 2013; Li et al., 2014). In addition, spontaneous locomotor activity in rats was not impaired by BNO at doses effective in inhibiting alcohol drinking, demonstrating that the suppression of alcohol drinking behavior cannot be ascribed to motor function impairment or sedation (Li et al., 2014). Finally, the CPP paradigm was used to evaluate the conditioned reinforcing effect of BNO in rats (Liu et al., 2012; Li et al., 2014). We found that BNO treatment at 70 mg/kg, an effective dose as demonstrated above, did not result in

either preference or aversion for the BNO-paired compartment, preliminarily suggesting that the effects on alcohol drinking likely do not result from interoceptive properties of BNO.

The metabolism of BNO appears to be complicated, as it is reported that BNO and brucine can transform into each other in circulating blood. BNO as a tertiary amine can be enzymatically dealkylated and structurally decomposed to secondary amines, while a proportion of BNO can be metabolically reduced into brucine by the enzyme aldehyde oxidase. Retrogradely, brucine can also be oxidized into BNO, catalyzed by cytochrome P450 and the monooxygenase with flavin, which is a critical detoxification method of alkaloids (Takekawa et al., 1997, 2001). *Chen* et al discovered that 65.4% brucine could be readily metabolized into the main metabolite BNO in rat liver tissue in a 2h incubation period via an *in vitro* metabolism study (Chen et al., 2012). Pharmacologically, BNO rather than brucine should be the main existent form for both brucine and BNO in circulating blood, as BNO is highly hydrophilic and soluble, and has a longer elimination period (Bickel et al., 1971; Ziegler & Gold, 1971; Rose & Castagnoli, 1983). Our speculation is that both BNO and brucine are active ingredients in ameliorating alcohol drinking phenotypes, because the critical structure that plays an active part in pharmacological functions are identical, while the coordinated oxygen

atom in the chemical structure of BNO failed to modify pharmacological activity, but lowered toxicity and increased hydrophilicity.

The mechanism of BNO to relieve alcohol drinking phenotypes remains to be elucidated. Both BNO and brucine can pass the brain blood barrier, and thus it is likely that their effects are exerted in the CNS (Cai et al., 2009). We propose that the effect of BNO is exerted via inhibitory glycine receptors, a type of inhibitory receptors extensively distributed in spinal cord and brain stem. *Gallegos* et al demonstrated that glycine receptors in dopamine receptor D1 receptor (D1R) expressed in medium spiny neurons are sensitive to small quantities of ethanol and further modulate the activity of nucleus accumbens, even at 5 mM (Gallegos et al., 2019). Meanwhile, the structure Loop 2 of a glycine receptor is considered a key structure and determines sensitivity to ethanol (Eggers & Berger, 2004; Crawford et al., 2007; Perkins et al., 2012). In addition, homomeric α1 or α2 GlyRs in Xenopus oocytes may be potentiated, while the activities of GlyR can be enhanced through prolonging the burst duration by ethanol (Mascia et al., 1996; Welsh et al., 2009; San Martin et al., 2016). Furthermore, BNO and brucine antagonize the α1 and α1β glycine receptors with Ki values of 1.4 and 1.7 μmol/L, respectively, in human embryonic kidney 293 cells (Jensen et al., 2006). It is postulated that BNO and the metabolite brucine can competitively antagonize the

glycine receptors to reduce ethanol-induced dopamine release in the nucleus accumbens, and normalize the maladaptive signaling in reward circuitry (Li et al., 2014). It has been found that the elimination half-life ($t_{1/2}$) of BNO and brucine in the blood are 5.7 and 1.04 h, respectively, in rats (Xu et al., 2003; Chen et al., 2009; Chen et al., 2011). Obviously, the post-treatment effect is attributed to the reparation of damaged circuits or the normalization of the neurotransmitter system by the BNO and brucine (Fig.2). However, the underlying neurobiology seems much more complex and awaits a deeper exploration, as some present findings appear in part contradictory to the proposed mechanism, i.e. that bilateral accumbal microinfusion of glycine reduced alcohol acquisition in Wistar rats (Molander et al, 2005). This result likely reflects the intense activation of NMDA receptors by overwhelmingly enhanced concentrations of glycine in the mesolimbic dopamine system, as glycine is a prerequisite co-agonist for the activation of NMDA receptors, which have a much higher density than glycine receptors (Irimia et al, 2017).

In summary, BNO selectively reduces alcohol drinking behavior in alcohol-preferring FH/Wjd rats, and is a promising candidate medication with higher safety, greater water solubility, longer elimination half-life and a larger therapeutic window. The homologue class of brucine may be a potential family for alcoholism therapy.

Acknowledgement

This work was supported by National Natural Science Foundation of China (81773705) and National Key Technology R&D Program of China (2011BAK04B08).

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615 **Figure Legends**
616 Figure 1 Chemical structures of brucine (a) and brucine N-oxide (b).

Figure 2 The effects of BNO treatment at 0, 30, 50 and 70 mg/kg (7 d, s.c., bid) on ethanol consumption, preference, water and food intake in alcohol-preferring FH/Wjd rats. (A and B) During the period of BNO injections, ethanol consumption and preference were significantly inhibited by BNO injections in rats (n = 9). (C and D) The increase in water intake was almost significant, though there was no significant overall effect, while food intake during BNO treatment did not differ among the groups. Test phases shown from left to right in the figure: the saline phase (day 1-3), the drug-treatment phase (day 4 - 10) and the post-treatment phase (day 11 - 13). * $p < 0.05$, ** $p < 0.01$ versus the saline group.

Figure 3 The effects of BNO treatment on the cumulative ethanol intake during the 7d injections in alcohol-preferring FH/Wjd rats. BNO treatment inhibited the cumulative ethanol consumption in rats (n = 9). * $p < 0.05$, *** $p < 0.001$ versus the saline group.

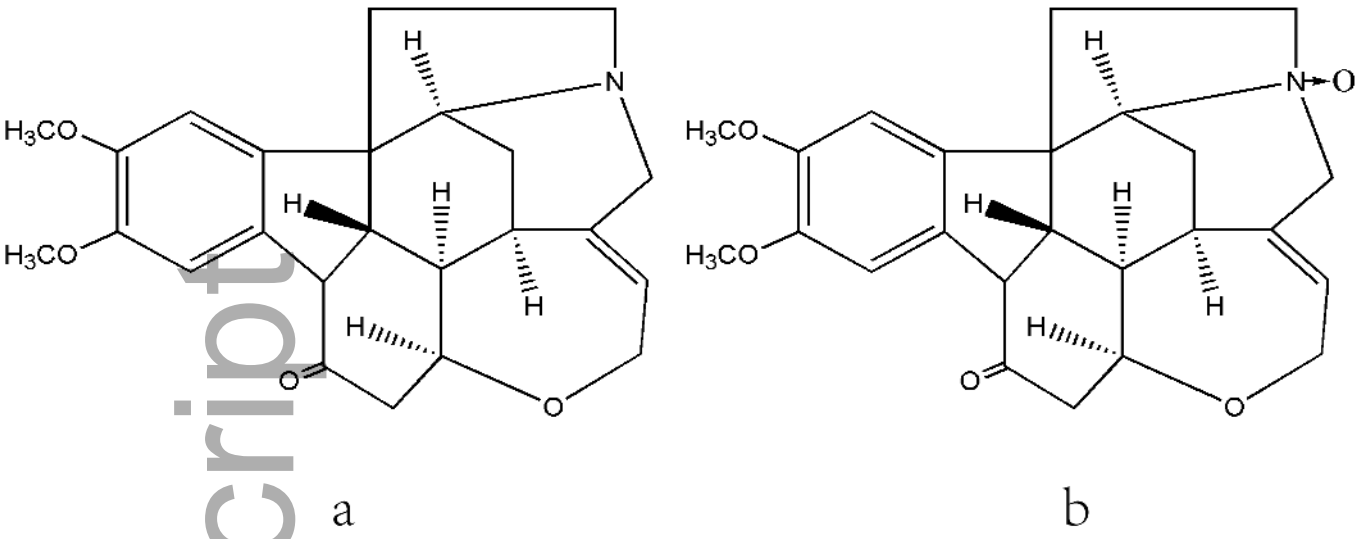
Figure 4 The effect of BNO treatment at 70 mg/kg (s.c.) on deprivation-induced ethanol consumption, preference and total fluid intake in alcohol-preferring FH/Wjd rats. Ethanol and water intake were monitored on d1 and d2 after the re-introduction. (A) 70 mg/kg BNO reduced ethanol intake on d1 and d2 compared to the saline group, while there was a significant ADE after a period of 4d deprivation in saline-treated rats (n = 8). (B) 70 mg/kg BNO

significantly reduced alcohol preference on d1 and d2 in rats. (C) The total amount of fluid intake did not differ among the groups. * $p < 0.05$ and ** $p < 0.01$ versus the saline group.

Figure 5 The effect of 70 mg/kg BNO (7 d, s.c., bid) on sucrose preference in FH/Wjd rats. BNO treatments did not change sucrose preference in rats (n = 6). Test phases shown from left to right in the figure: the saline phase (day 1- 3), the drug-treatment phase (day 4 - 10) and the post-treatment phase (day 11 - 13).

Figure 6 The effect of BNO treatment at 0, 30, 50 and 70 mg/kg (s.c.) on locomotor activity in FH/Wjd rats. BNO treatment did not alter spontaneous locomotor activity in rats (n = 8).

Figure 7 The effect of BNO treatment at 0, 30, 50 and 70 mg/kg (s.c.) on the CPP scores in FH/Wjd rats. BNO injections did not induce rewarding or aversive effects in rats (n = 6). Saline and morphine groups were used as control groups. ** $p < 0.01$ versus the saline group.



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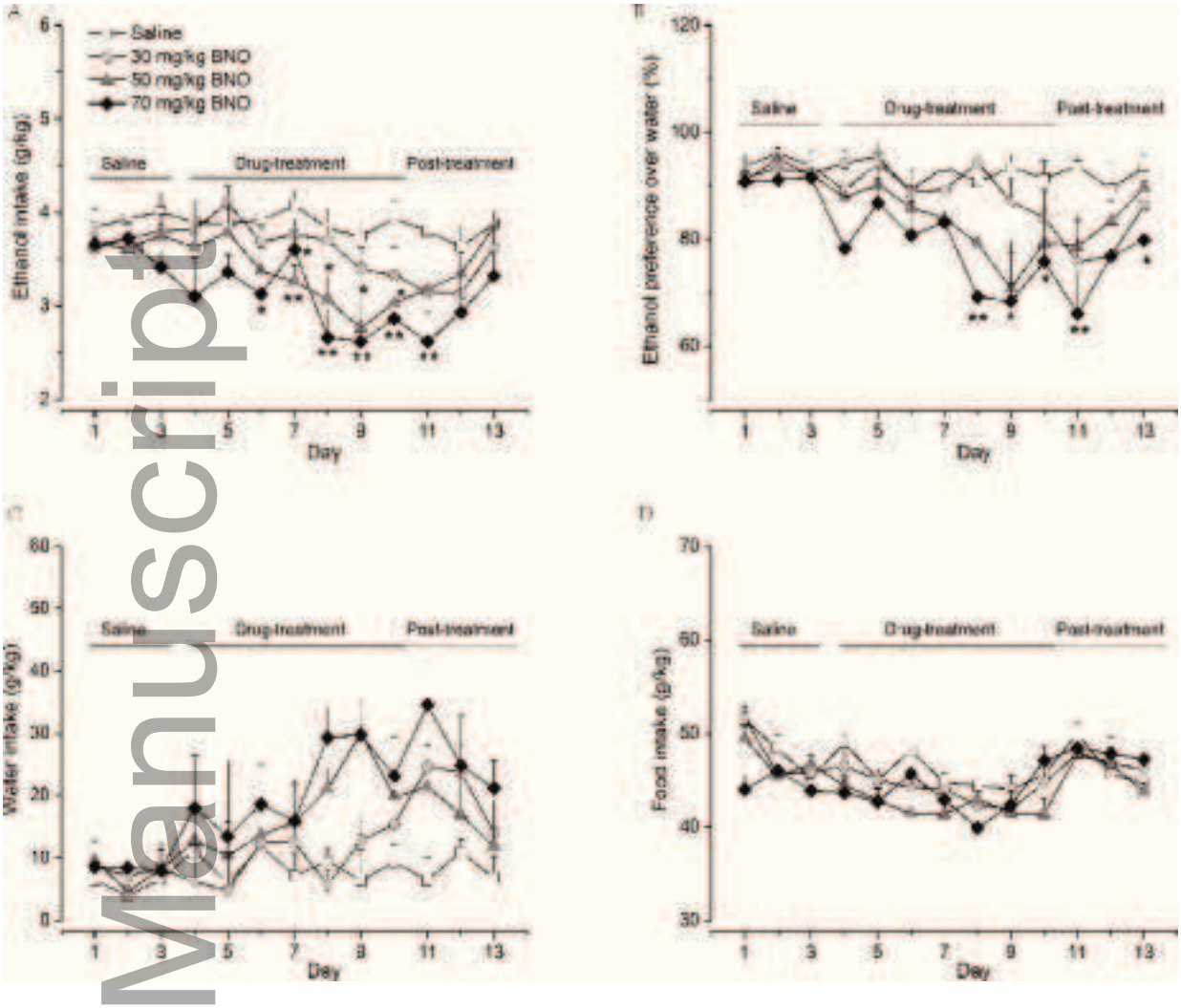

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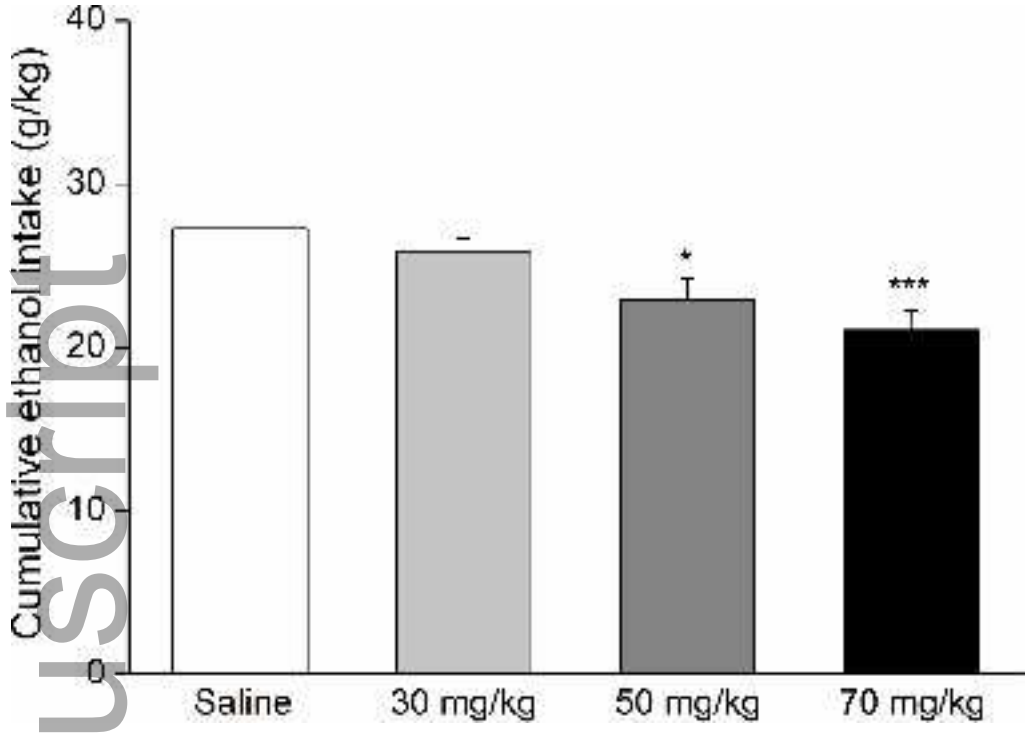

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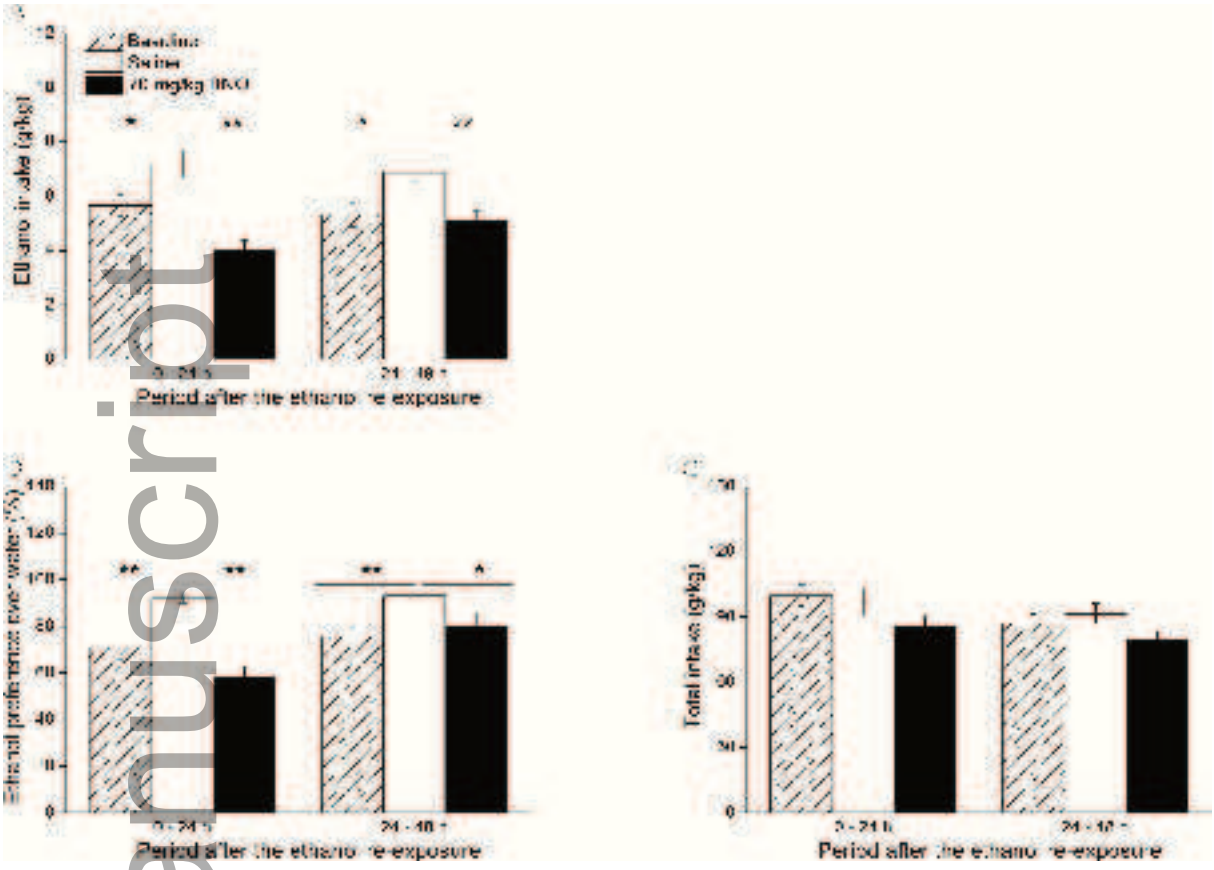

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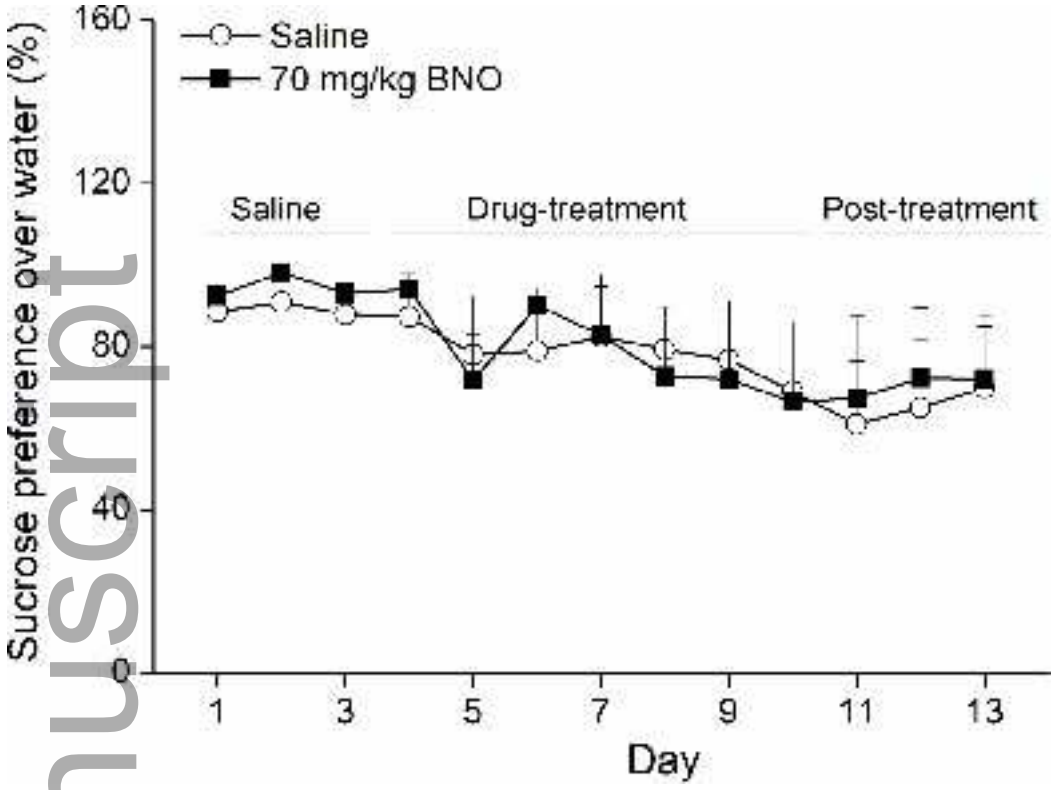

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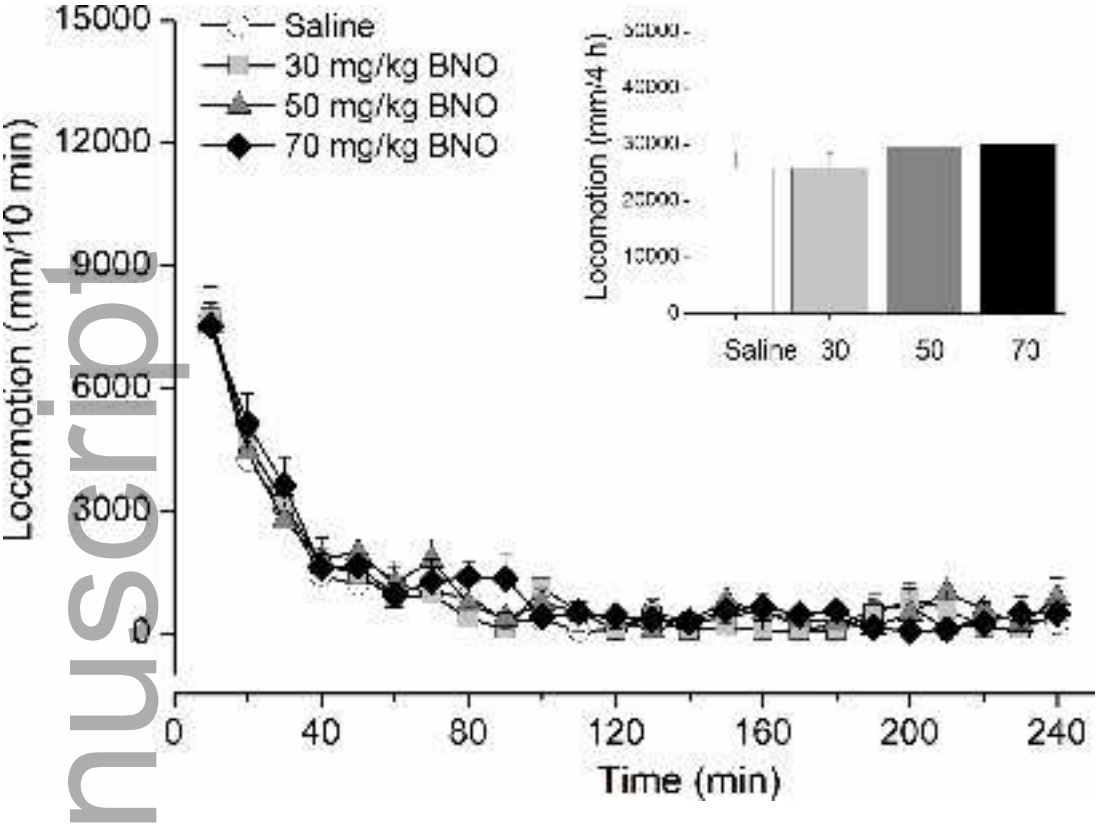

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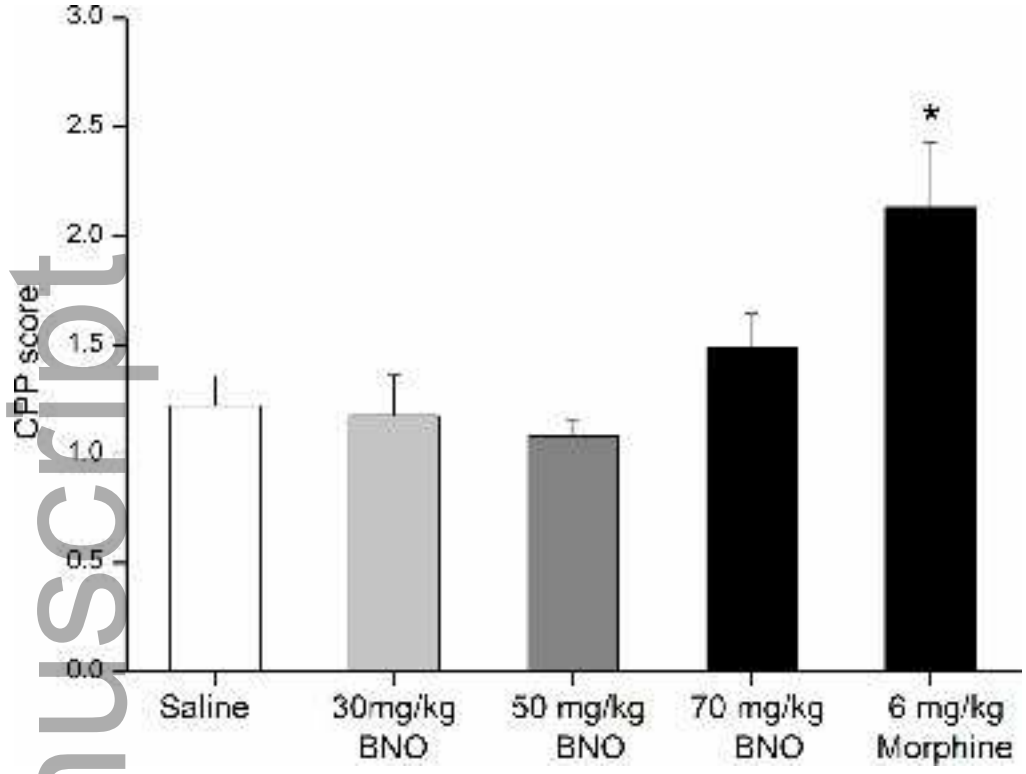

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Title:
Brucine N-Oxide Reduces Ethanol Intake and Preference in Alcohol-Preferring Male Fawn-Hooded Rats

Date:
2020-05-17

Citation:
Wei, S., Li, Y. -L., Gong, Q., Liang, H., Liu, Q., Bernardi, R. E., Zhang, H. -T., Chen, F., Lawrence, A. J. & Liang, J. -H. (2020). Brucine N-Oxide Reduces Ethanol Intake and Preference in Alcohol-Preferring Male Fawn-Hooded Rats. ALCOHOLISM-CLINICAL AND EXPERIMENTAL RESEARCH, 44 (6), pp.1321-1328. https://doi.org/10.1111/acer.14344.