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Nucleus Accumbens Shell Orexin-1 Receptors Are Not Needed For Single-Bottle Limited Daily Access Alcohol Intake in C57BL/6 mice

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Abstract

Excessive, binge drinking is a major contributor to the great harm and cost of alcohol use disorder. We recently showed, using both limited and intermittent access two-bottle-choice models, that inhibiting nucleus accumbens shell (Shell) orexin-1-receptors (Ox1Rs) reduces alcohol intake in higher-drinking male C57/BL6 mice (Lei et al., 2019). Oher studies implicate Ox1Rs, tested systemically, for several higher-drinking models including the single-bottle, Rhodes Drinking-in-the-Dark paradigm. Here, we report studies examining whether Shell Ox1Rs contribute to alcohol intake in male mice using a single-bottle Limited Daily Access (LDA) LDA drinking model modified from drinking-in-the-dark paradigms (2-hour access starting 3 hours into the dark cycle, 5 days per week). Also, some previous work has suggested possible differences in circuitry for one- versus two-choice behaviors, and thus other mice first drank under a single-bottle schedule, and then an additional water bottle was included two days a week starting week 3. Surprisingly, at the same time we were determining Ox1R importance for two-bottle-choice models, parallel studies found that inhibiting Shell Ox1Rs had no impact on drinking using the single-bottle LDA model, or when a second bottle containing water was added later during drinking. Furthermore, we have related Shell Ox1R regulation of intake to basal consumption, but no such pattern was observed with single-bottle LDA drinking. Thus, unlike our previous work showing the importance of Shell Ox1Rs for male alcohol drinking under several two-bottle-choice models, Shell Ox1Rs were not required under a single-bottle paradigm, even if a second water-containing bottle was later added. These results raise the speculations that different mechanisms could

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Author contributions

KL and CK contributed to conception and design of the study, data acquisition, analysis and interpretation, and preparation of manuscript. FWH contributed to conception and design of the study, data analysis and interpretation, and preparation of manuscript.

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promote intake under single- versus two-bottle access conditions, and that the conditions under which an animal learns to drink can impact circuitry driving future intake.

Keywords

alcohol; addiction; orexin; model; differences

INTRODUCTION

Alcohol Use Disorder (AUD) extracts substantial costs, and excessive, binge-like alcohol drinking is both a strong impediment to treating AUD (Harwood, Fountain & Livermore, 1998; Larimer, Palmer & Marlatt, 1999; Blincoe et al., 2002; Mokdad, Marks, Stroup & Gerberding, 2004; Dawson, Grant & Li, 2005; Hingson, Heeren, Winter & Wechsler, 2005; Rehm et al., 2009; Koob & Volkow, 2010; Bouchery et al., 2011; Sacks et al., 2013; CDC, 2014; SAMHSA, 2014) and also a major contributor to the harm and costs associated with AUD (e.g., CDC, 2014). Thus, it is important to identify brain and molecular mechanism that promote binge intake, which could improve AUD treatment (Spanagel, 2009; WHO, 2014). In particular, a number of animal models have been developed to help uncover these critical underlying mechanisms, with a particular focus on paradigms with higher drinking levels to better reflect binge intake in humans (see Thiele & Navarro, 2014).

In seeking the mechanisms that promote excessive alcohol drinking, recent work from our lab (Lei et al., 2016a; 2016b; 2019) and others (Anderson et al., 2014; Mahler et al., 2014; Olney, Navarro & Thiele, 2015; Alcaraz-Iborra et al., 2017; Moorman, James, Kilroy & Aston-Jones, 2017) has identified orexin receptor signaling as an important driver of alcohol consumption. In particular, orexin-1 receptors (Ox1Rs) often play a central role in highly motivated behaviors directed towards additive substances such as alcohol, as well as to higher-value natural rewards (Borgland et al., 2009; Moorman & Aston-Jones, 2009; Cason et al., 2010; Anderson et al., 2014; Baimel et al., 2014; Mahler et al., 2014). As a result, Ox1Rs have been considered to represent a possible therapeutic target to treat AUD and related conditions (Khoo & Brown, 2014; Li, Jones & de Lecea, 2016).

We recently examined how alcohol consumption is regulated by Ox1Rs in the nucleus accumbens shell (Shell), a region important for a number of motivated and addiction-related behaviors (Anderson et al., 2008; Chaudhri, Sahuque, Schairer & Janak, 2010; Saddoris, Sugam, Cacciapaglia & Carelli, 2013; Castro, Cole & Berridge, 2015; Corbit & Balleine, 2016; Marchant, Kaganovsky, Shaham & Bossert, 2015; Millan et al., 2015) including alcohol intake (Kasten & Boehm, 2014; Lum, Campbell, Rostock & Szumlinski, 2014; Wilden et al., 2014; Ramaker et al., 2015). Specifically, we found that Shell Ox1Rs are critical for driving alcohol drinking in male C57BL/6 mice consuming alcohol under either Limited Daily Access (LDA), a two-bottle-choice variant of alcohol drinking-in-the-dark (Lei et al., 2016b; 2019), which leads to binge-level blood alcohol concentrations (Lei et al., 2016a), or during two-bottle-choice intermittent access to alcohol (24-hr access three days per week) (Lei et al., 2019). We also discovered that Shell Ox1R enhancement of alcohol drinking occurs within higher-drinking individuals, with little impact in more

moderate drinkers (Lei et al., 2019), in agreement with previous findings that Ox1Rs (tested systemically) primarily promote alcohol intake in higher drinkers (Moorman & Aston-Jones, 2009; Alcaraz-Iborra et al., 2017; Moorman, James, Kilroy & Aston-Jones, 2017). Thus, our findings suggest that Shell Ox1Rs are important for binge alcohol intake in higher-drinking male mice under different two-bottle choice paradigms.

During the same period when we were testing Shell Ox1R importance for alcohol intake under two-bottle choice LDA and intermittent access paradigms, we also examined in parallel the impact of Shell Ox1R inhibition with SB-334867 (SB), a widely used Ox1R antagonist (Hollander et al., 2008; Espana et al., 2010; Plaza-Zabala, Flores, Maldonado & Berrendero, 2012; Qi, Wei, Li & Sui, 2013; Brown et al., 2015; Lei et al., 2016b), on alcohol consumption under a single-bottle LDA model, modified from the Rhodes drinking-in-the-dark paradigm (Rhodes et al., 2005). Previous studies show that systemic Ox1R inhibition reduces single-bottle drinking-in-the-dark intake (Anderson et al., 2014, see Discussion). Here, we find that blocking Shell Ox1Rs had no impact on alcohol drinking during a single-bottle access to alcohol LDA model. Further, Shell Ox1Rs were not required when a second bottle with water was added concurrently later in training. Thus, in concert with some previous studies (described in Discussion), our findings support the speculative possibility that some aspects of brain signaling that promote alcohol drinking could differ among two-bottle- versus one-bottle-choice restricted-access intake models.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were purchased from Jackson Laboratories at 6–8 weeks old. After ~2 weeks of habituation to the housing colony, mice were allowed to drink alcohol (methods below). All procedures followed the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, and with approval of the Institutional Animal Care and Use Committee of UCSF. Animals in the present study were not included in any previous study. Mice from two-bottle LDA studies showing Shell Ox1R importance for alcohol intake in higher-drinking mice (published in Lei et al., 2019), which were conducted in parallel with the present work, serve as positive controls for the present findings.

Alcohol drinking

For experiment A, mice had access to drink a 20% (w/v) alcohol for 2 hours starting 3 hours into the dark cycle (Fig.1). During this time, only the single bottle (containing alcohol) was present). Animals otherwise had food and water *ad libitum*. For experiment B, mice drank alcohol from a single bottle for the first two weeks; starting week 3 of alcohol drinking, a second bottle (containing water) was present on two drinking days (randomized) within each week (Fig.1). Presence of the second bottle during weeks 3 and 4 allowed habituation to the novelty of a second bottle, and the second, water-containing bottle was then present for each experimental test session. These studies did not use water deprivation.

We note that the duration and timing of pre-drinking acclimation to vivarium, pre-surgery drinking, surgical methods, and post-surgery drinking before testing were all identical to

methods used in previous studies (Lei et al., 2016b; 2019), as were randomization and testing schedule across weeks and method for determining amount consumed, the only exception being the drinking model. Thus, our findings here are directly comparable to parallel two-bottle drinking studies in Lei et al., 2019, where intra-Shell SB reduced two-bottle choice LDA drinking levels, which thus serve as a positive control to the present studies. Also, the average alcohol drinking levels on the vehicle days were 2.56 ± 0.27 g/kg for Experiment A, 2.24 ± 0.24 g/kg for Experiment B, and 1.78 ± 0.10 for LDA with SB or muscimol/baclofen Shell injections in Lei et al. (2019) (n=25); one-way ANOVA was $F_{(2,45)}$=5.492, p=0.0073, although only Experiment A was different from Lei et al. (2019) data by post-hoc (p=0.0071), while Experiment B was not (p=0.1675).

Surgery and intracranial injection

Methods were the same as previously described (Lei et al., 2019). After ~2-wk of alcohol access, surgery was performed to bilaterally implant guide cannulae (Plastics One) targeting the NAc Shell (AP +1.5, ML ±0.5, and DV −4.8mm), where coordinates are relative to Bregma from skull surface, and both cannulae are in the same implant. After surgery, mice were allowed to recover for at least 3 days before alcohol drinking was resumed. Before experimental testing began, mice received 5 days of 2–3 mins per day habituation to handling. Animals then had the first intracranial injection of saline, to habituate to injection. Thereafter, the Ox1R inhibitor SB (3 μg/side: Hollander et al., 2008; Espana et al., 2010; Plaza-Zabala, Flores, Maldonado & Berrendero, 2012; Qi, Wei, Li & Sui, 2013; Brown et al., 2015; Lei et al., 2016b) versus vehicle (100% DMSO, see Lei et al., 2019 for discussion and other references) were tested within each animal with a randomized, counterbalanced design. For Experiment A, each animal received SB and vehicle once. For Experiment B, each animal received both SB and vehicle twice, in a randomized manner across mice, and the two values for each condition were averaged to give a single value for vehicle and drug exposure for each animal. This is what we have done in previous studies (Lei et al., 2016a; 2016b; 2019) to reduce the impact of variability, and was done here to reduce the possibility that a lack of significant effects of intra-Shell Ox1R inhibition might be due to variability in the drinking data.

Histology was performed as in Lei et al., 2016b; 2019. Mice were perfused intracardially with paraformaldehyde, then cut into 50 μM sections frozen on the cryostat. Sections were stained with cresyl violet, mounted, and the placement determined by the most ventral cannula position. One mouse each from Experiments A and B were removed due to incorrect placement. The exclusion criterion was when one of the cannulae was in the septum (and since the two cannulae are linked in the same implant, the other cannulae was usually in the NAc core). Most mice for studies here were on target, which likely reflects where we have performed a great number of intra-Shell injections in mice (>150 across Lei et al., 2016b; 2019).

For intracranial injection, SB was diluted in 100% DMSO. While 100% DMSO is a higher dose for intracranial injections, we (Lei et al., 2016b; 2019) and others (Pierce, Pierce-Bancroft & Prasad, 1999; James et al., 2011; Simms et al., 2011; Vendruscolo et al., 2015) have used this dose as an intracranial vehicle and shown that it does not have non-

specific effects. For example, our studies were performed using a randomized, Latin-squares design, and included alcohol-only drinking on days in between the intracranial test sessions. Any possible lingering toxicity of DMSO should alter alcohol intake on days after DMSO exposure, but this was not observed (see Lei et al., 2016b).

Data analyses

Methods were the same as previously described (Lei et al., 2019). Briefly, alcohol intake was determined by weighing the alcohol bottles, and converting to g/kg of body weight. Drinking level was analyzed using Prism (GraphPad) and paired t-tests were used to compare the drinking level on vehicle vs drug sessions within each animal. We did not include order in our analyses. For illustrative purposes, Tables 1 and 2 show drinking data on days before and in between test days, and on test days, for the two Experiments.

For analyzing basal intake (intake on vehicle injection days, as we did in Lei et al., 2019; Wegner et al., 2019) when compared with change in intake during SB infusion, we followed our previous work (Lei et al., 2019) and expressed the change in drinking with Shell SB as log[100*(intake during drug treatment)/(intake during vehicle)], followed by a regression analysis (Prism). Using this measure, a log value of 2 (log[100]) indicates no intake change with treatment. As discussed in greater detail in Lei et al., 2019, we did this for several reasons. There are several challenges when calculating drug-related changes in intake as a percent changes in drinking (e.g. showing a change from 3 g/kg under vehicle to 1.5 g/kg under drug as a 50% drop in drinking). First, drinking in mice shows a high level of variability, e.g. Fig. 5 of Lei et al. (2019) which shows that alcohol drinking on the first intake day is highly correlated with consumption levels in the subsequent weeks, and yet there is much variability. In particular, if an animal has lower basal drinking, then using percent change can yield very large percent changes (especially increases in drinking) simply due to variability. In contrast, higher basal drinking is less likely to show this problem. Since we were strongly interested in examining the impact of Shell Ox1R inhibition in relation to basal drinking levels, in Lei et al., 2019, we developed this log transformed method which somewhat reduces the variability. Even so, we note that log transforming is a compromise, with Lei et al. (2019) discussing the strengths and weaknesses of each method (simple percent change in drinking versus log transformed), although Fig. 2 of Lei et al., 2019, shows that the two analysis methods yield similar results (although with less variability with the log transformed method). However, Lei et al. (2019) comparisons each involved >100 animals, and the reduced variability with the log transformed method is likely to increase the ability to observe basal intake related changes in smaller sample sizes.

RESULTS

In Experiment A, we examined whether Shell Ox1R inhibition with SB would alter single-bottle LDA alcohol intake (Fig.1). However, SB infusion in the Shell had no impact on alcohol drinking levels (Fig.2A; n=12; paired t-test, t_{11}=0.4075, p=0.6914), although similar Shell Ox1R inhibition does reduce alcohol consumption in several two-bottle-choice models

(Lei et al., 2016b; 2019). Thus, Shell Ox1R inhibition did not regulate single-bottle alcohol drinking. Histology validating intra-Shell placement is shown in Fig.2B.

To examine whether placing a second bottle (containing water) during the alcohol drinking session might be required to activate Ox1R-dependent mechanisms, we modified the drinking paradigm. In particular, in Experiment B, a separate group of mice primarily drank alcohol using a single-bottle method as in Experiment A. However, starting week 3 of drinking, a second bottle (containing water) was present on two drinking days within each week, including during each experimental test session (Fig.1). Under these conditions, inhibition of Shell Ox1Rs still had no effect on alcohol consumption levels (Fig.3A; n=11; paired t-test, t_{10}=0.2178, p=0.8320; histology shown in Fig.3B). Thus, placing a second, water-containing bottle in addition to the alcohol bottle did not recruit Shell Ox1R-dependent mechanisms to mediate alcohol drinking. Also, there was no difference in drinking, determined on vehicle test day(s) in Experiments A and B (t_{21}=0.8840, p=0.3867), showing that adding a second bottle (containing water) did not alter alcohol intake levels (see also Tables 1 and 2).

We next sought to determine whether Shell Ox1R inhibition might have different effects on drinking depending on the basal intake level (determined by intake level on vehicle injection days, as we did in Lei et al., 2019; Wegner et al., 2019). Previous work from our lab (Lei et al., 2019) and others (Moorman & Aston-Jones, 2009; Alcaraz-Iborra et al., 2017; Moorman, James, Kilroy & Aston-Jones, 2017) demonstrates that Ox1Rs predominantly promote alcohol intake in higher-drinking individuals, with little net impact in moderate-binging mice (Lei et al., 2019). To observe a clear relation between basal intake and Shell Ox1R impact on drinking, previous studies combined results across drinking conditions and groups to give large data sets (Lei et al., 2019). For example, the control group in Lei et al. (2019), taken from a number of conditions with no average change in drinking with treatment, did show a significant correlation between basal intake and impact of drug treatment, although the slope in this control group was much smaller (−0.2428) than seen in the Shell Ox1R and global inhibition group (−0.8445). This small (but significant) relationship in control animals is likely related to regression to the mean, where it is easier for larger values to go down and smaller values to go up, rather than an actual effect on intake (discussed further in Lei et al., 2019).

Thus, here we combined data from our two Experiments and examined the relationship between basal alcohol intake level and impact of infusing SB in the Shell. For these analyses, we expressed the change in drinking with drug versus vehicle with a log transformed metric, as in Lei et al., 2019; the rationale for this is detailed in Methods. Overall, there was no relation between basal intake levels and the impact of intra-Shell SB on alcohol consumption (Fig.4; p=0.3266, slope=−0.0729). With one outlier removed (where SB did drop drinking in one mouse in Experiment A), there was a significant relationship between basal intake and impact of drug infusion (p=0.0018), but the slope was quite low (−0.1646), even lower than the slope of the control group (−0.2428) in Lei et al. (2019). These data support the suggestion than Shell Ox1R inhibition had no overall impact on alcohol drinking, even if effects might be apparent in a few animals.

DISCUSSION

At the same time that we were examining the importance of Shell Ox1Rs for two-bottle-choice models (Lei et al., 2016b; 2019), we also determined whether Shell Ox1Rs contribute to single-bottle LDA alcohol drinking. Unlike our two-bottle drinking models, where studies in Lei et al., 2019, were performed in parallel and reflect “positive controls” for the present work, we found that inhibiting Shell Ox1Rs had no impact on alcohol drinking during single-bottle LDA intake. Furthermore, Ox1R inhibition in the Shell also had no behavioral impact in a separate experiment where a second bottle (with water) was added during later alcohol consumption sessions. Finally, analysis of basal alcohol drinking versus the impact of Shell Ox1R inhibition also suggested no impact of intra-Shell SB across mice whose drinking training began with single-bottle access to alcohol. Thus, our results concur with other findings (below) that raise the presently speculative possibility that different brain circuits may be recruited depending on the nature of choice during the task, in our case the requirement, or lack thereof, for Shell Ox1Rs. We also speculate that the conditions under which an animal learns to drink can impact the brain circuitry that drives future intake, at least in some rodent drinking models.

There are some notable limitations for our studies. We did not measure blood alcohol levels in these studies, and thus some caution is warranted in terms of inferring whether pharmacological levels of alcohol were achieved, although our previous studies with other drinking models with equivalent duration of intake and lower intake levels do show binge levels of blood alcohol (Lei et al., 2016a). In addition, the drinking levels under our single-bottle choice models are higher than those we observe under two-bottle choice models, but are lower than those seen by other groups using the two-hour single-bottle LDA alcohol drinking model (Rhodes et al., 2005; Thiele & Navarro, 2014). The reason for this remains unclear, and thus our single-bottle studies are best interpreted in relation to our own two-bottle-choice findings showing the importance of Shell Ox1Rs for higher-drinking male mice (Lei et al., 2016a; 2019). We do note that our single-bottle intake was ~20–30% higher than what we have observed for two-bottle choice intake (see Methods, and Lei et al., 2016a; 2016b; 2019). However, our parallel studies find that Shell Ox1Rs drive alcohol consumption predominantly within higher-drinking individuals under two-bottle choice LDA conditions (Lei et al., 2019). Thus, these findings indicate the possibility that differences in alcohol intake levels were less likely to explain a lack of Shell Ox1R role for single-bottle LDA consumption studied here. In agreement, we found little relation between basal alcohol intake and Shell Ox1R inhibition. Also, we note that results from Experiment B were averages of two tests (as in our previous studies), whereas results from Experiment A were from a single treatment. Since SB had very little effect in either Experiment, and the average drinking levels across the two Experiments were not different, we consider it unlikely that the single versus multiple test of SB was responsible for the lack of effect of SB in Experiment B.

We note that our studies are consistent with other findings suggesting the speculative possibility of different brain mechanisms contributing to limited-access two-bottle vs one-choice alcohol intake. For example, knockout of the PKC isoform PKM alters intermittent (24 hours) access and limited daily access with two-bottle choice, but does not change

single-bottle drinking-in-the-dark intake (Lee et al., 2014). In addition, modulation of the neuro-immune molecule TLR2 impacts two-bottle but not one-bottle drinking-in-the-dark consumption in mice (Blednov et al., 2017). Interestingly, other recent studies have addressed the presence of choice in alcohol and other addiction related animal models (Augier et al., 2018). Further, Anderson and McClearn (1981) bred mice for high and low alcohol acceptance under single-bottle conditions, and that the higher preference under one-bottle in the alcohol-preferring line was unrelated to alcohol preference under two-bottle conditions. More generally, different mechanisms have been observed when comparing one versus two main response options. For example, the orbitofrontal cortex is needed to avoid LiCl-conditioned saccharin (conditioned taste aversion) when a second, water bottle is present, while this region is not needed for avoiding when only aversion-paired saccharin is present (Ramirez-Lugo, Penas-Rincon, Angeles-Duran & Sotres-Bayon, 2016). Furthermore, the subthalamic nucleus is needed for the faster responding under single choice, without impacting the slower response with two choices (Baunez et al. 2001). Thus, there is precedent for the possibility that different brain circuits can mediate motivated action with one versus several choices. However, it has been noted that animals still are exercising a choice when presented with a single bottle of alcohol, as some mice strains choose not to drink under single bottle (Thiele & Navarro, 2014). Many additional studies will be needed to better understand the implications of possible differences in brain mechanisms between one-bottle and two-bottle choice alcohol drinking models, including the speculation that high-motivation signaling and brain regions, typified by Shell and Ox1Rs, may not be needed under simpler conditions. Additionally, systemic inhibition of Ox1Rs with SB does reduce single-bottle LDA alcohol consumption in C57BL/6 mice (e.g. Anderson et al., 2014), suggesting that Ox1Rs are required for single-bottle drinking, just not Ox1Rs in the Shell. However, we also note that high doses of SB are required systemically for this effect (with 30 but not 10 mg/kg effective at reducing alcohol drinking in mice in Anderson et al., 2014), and some studies have called into question the specificity of these higher SB doses (Gotter et al., 2012; Perrey & Zhang, 2018; Rodgers et al., 2001; reviewed in Hopf, 2020).

Thus, based on our findings and some previous work, we propose the possibility that the mechanisms which promote single-bottle intake may differ from those mediating two-bottle-choice drinking. However, our Experiment B shows that adding a second bottle with water, thus leading to a choice during test sessions, does not lead to Shell Ox1Rs becoming necessary for driving drinking. One speculative possibility is that the conditions under which an animal learns to drink can impact the brain circuitry that drives future intake. This may result from different circuits being recruited if drinking is learned under one versus multiple choice conditions. Another possibility, not mutually exclusive, is that the presence of the water bottle during early learning endows it with higher salience (as the bottle to ignore), while a water bottle added later does not gain such attention since the alcohol bottle is already learned to be of primary importance. We note that all such possibilities are highly speculative at present, and that alternate possibilities may also be important, although at present these remain unclear. However, we note that mice exposed to alcohol-only can quickly learn to express quinine-resistant intake (Lesscher, van Kerkhof & Vanderschuren, 2010; Lei et al., 2016c), and brain adaptations can occur in relation to a single alcohol

drinking session (Beckley et al., 2016; Wolstenholme et al., 2011). In addition, mechanisms for learning of place preference can strongly differ depending on the presence of a choice or not (Ramirez-Lugo, Penas-Rincon, Angeles-Duran & Sotres-Bayon, 2016). However, considerable future studies would be required to uncover possible behaviorally-relevant neuro-adaptations that occur under different initial drinking conditions (in this case, single-bottle versus two-bottle-choice) and impact subsequent intake

In conclusion, we show that Shell Ox1Rs are not required in C57BL/6 male mice for driving single-bottle LDA alcohol drinking, or for promoting alcohol consumption in mice trained to drink under single-bottle but where a second, concurrent bottle of water is added later. This is unlike previous studies showing that Shell Ox1Rs regulate several forms of limited-access, two-bottle choice alcohol intake. We also found no relation between basal intake and the impact of Shell Ox1R inhibition. Together, our studies provide new information about the speculative possibility that different brain circuits and molecules could be recruited depending on the alcohol drinking model used.

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Highlights

- Excessive drinking contributes strongly to the great harm of alcohol use disorder.
- Previous work: NAc Shell Ox1Rs mediate two-bottle alcohol intake in higher drinkers.
- Here, Shell Ox1Rs did not regulate single-bottle alcohol drinking.
- Shell Ox1Rs did not become important when a second water bottle was added.
- We speculate that early drinking conditions can impact circuitry of future intake.

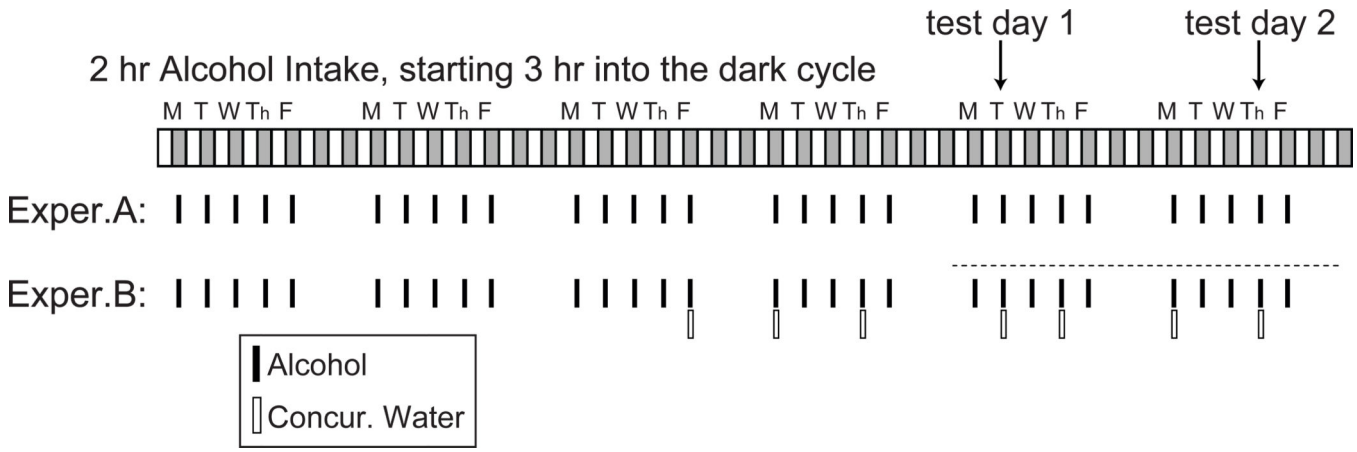

Figure 1. Cartoon example of drinking paradigms.

In Experiment A, animals drank alcohol from a single bottle for 2 hours, starting 3 hours into the dark cycle. Drinking in Experiment B was similar to A, except that a second bottle containing water was available concurrently during the test session on two days of each week (randomized) starting the 3rd week of drinking. In addition, in Experiment B, mice underwent a second round of vehicle vs drug injection (in a randomized manner), as described in greater detail in Methods; thus, the two weeks indicated by the dotted line were repeated twice in Experiment B. We note that Tuesday/Thursday are shown in this example for purpose of illustrating vehicle/drug injection test days, although other weekdays were also used. M, T, W, Th, and F indicate days of the week.

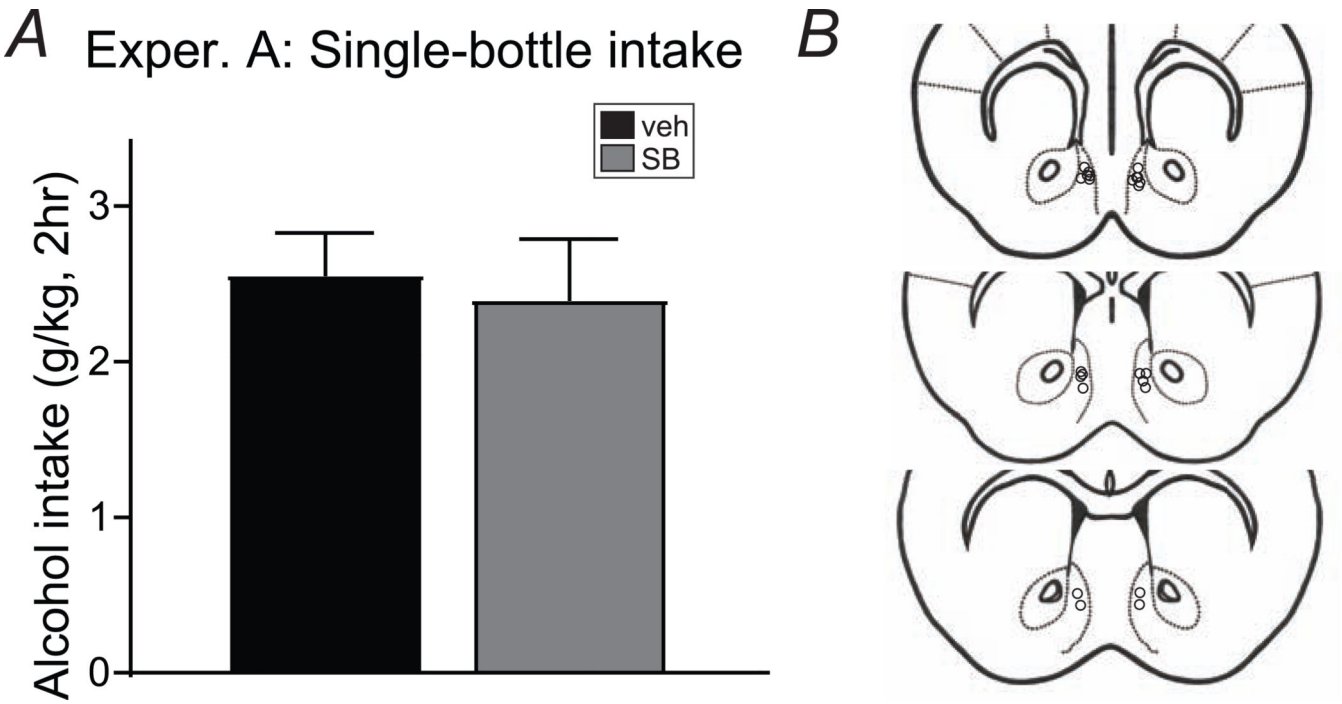

Figure 2. Ox1R blockade within medial NAc Shell did not reduce single-bottle, drinking-in-the-dark alcohol consumption in male C57/BL6 mice.

(A) Intra-Shell infusion of SB did not alter single-bottle LDA alcohol drinking. ***(B)*** Histology of placements in Shell.

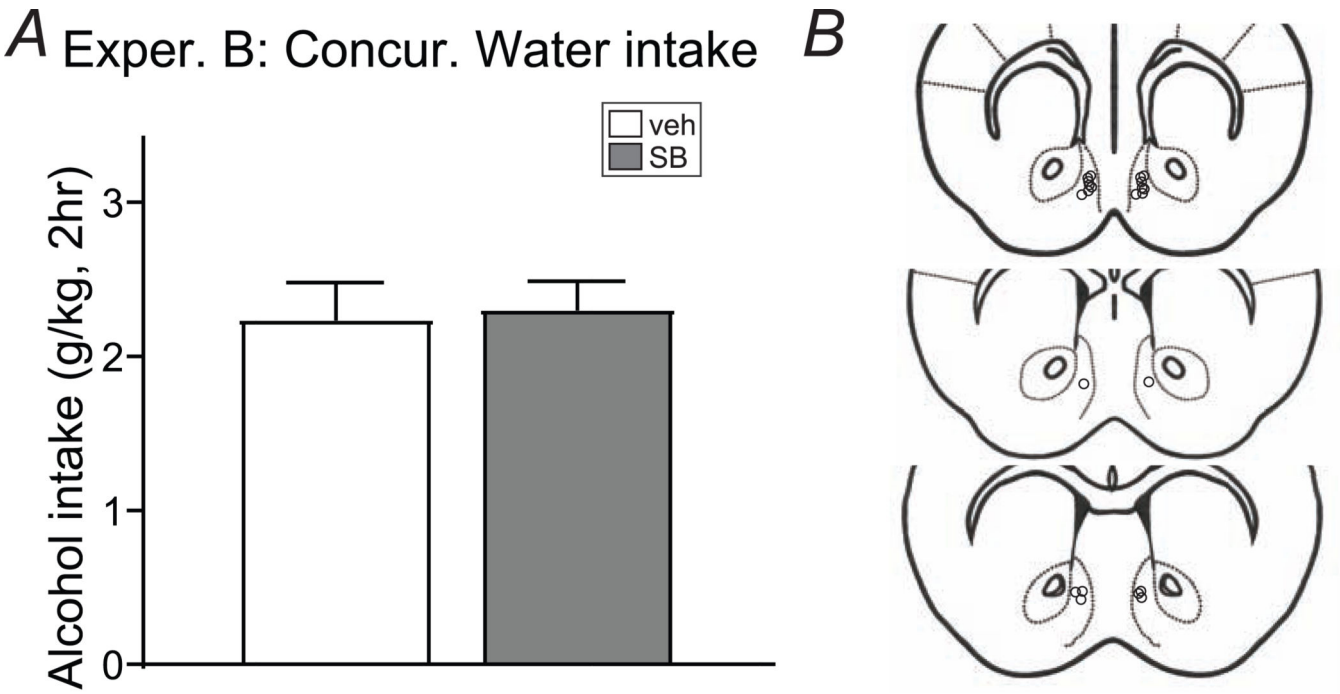

Figure 3. Shell Ox1R blockade did not reduce intake when a second, water-containing bottle was added later in training.

(A) Intra-Shell infusion of SB did not alter alcohol drinking in mice originally trained on single-bottle LDA alcohol consumption, but tested in the presence of a second bottle (containing water). ***(B)*** Histology of placements in Shell.

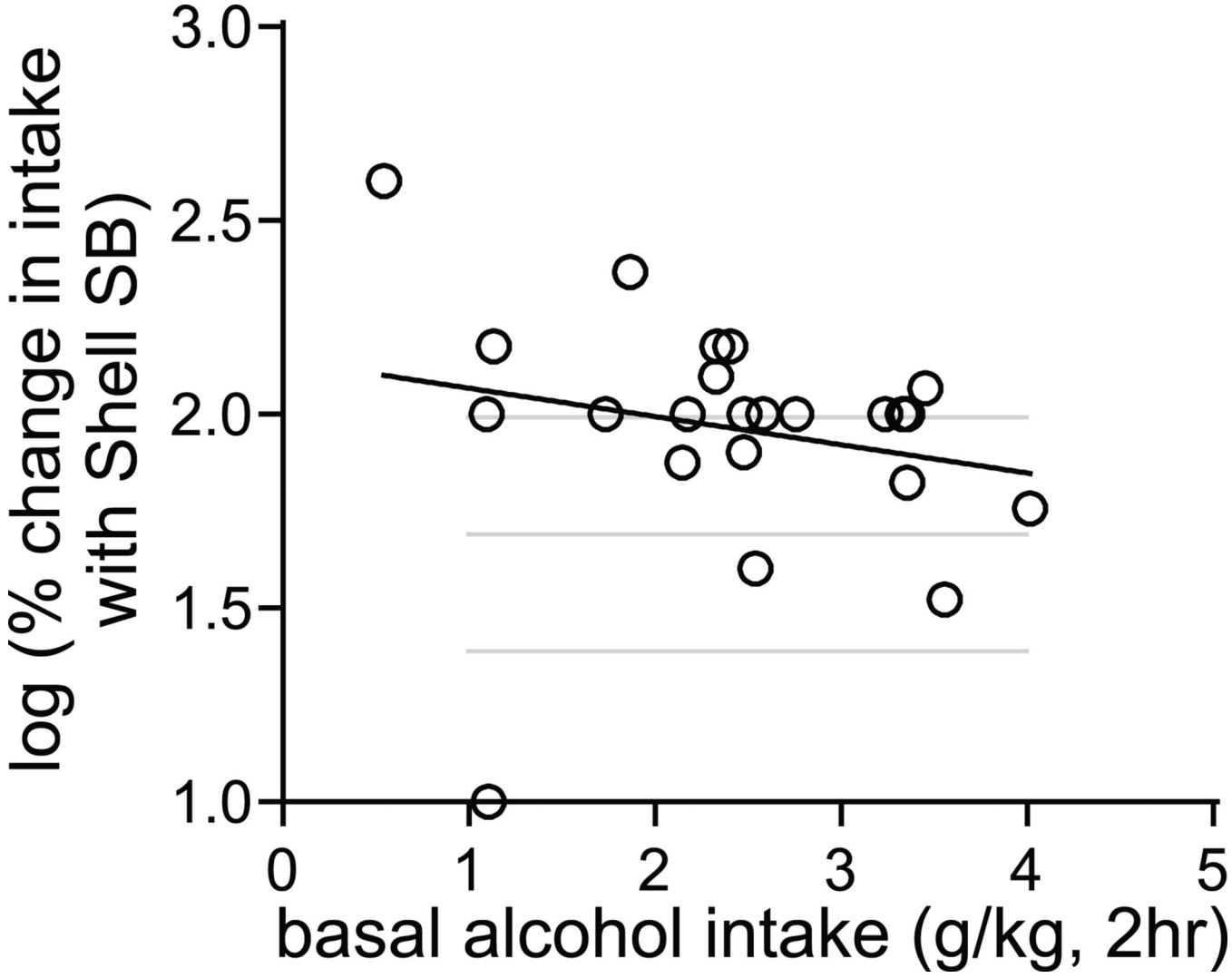

Figure 4. The impact of Shell Ox1R inhibition was minimally related to basal alcohol intake levels across individual mice.

Data were combined from Experiments A and B, as done before (Lei et al., 2019) to give a larger sample for correlating basal drinking level with possible changes related to intra-Shell SB infusion. There was no significant relationship. However, while there was a significant relation between basal intake and intra-Shell infusion when a single outlier was removed, the slope was still low; this likely reflects regression to the mean (see Results and Lei et al., 2019). Light gray bars are included to clarify, for the log scale, where 0% change (top line), 50% change (middle line) and 75% change (bottom line) in drinking level are represented.

Table 1.

Raw data for Experiment A. Alcohol drinking is given in g/kg. Data in bold/italics on test days were DMSO injection, while regular font on test days were SB injection. Drinking for non-test-days were average of 2–3 days of intake between the test days.

animal number	non-test-day drinking	test day 1	non-test-day drinking	test day 2
1	3.53	***1.87***	3.74	4.36
2	1.86	3.36	3.36	***3.36***
3	2.69	***3.45***	2.30	4.03
4	2.17	1.19	3.26	***3.56***
5	2.75	***2.58***	3.61	2.58
6	1.69	1.02	2.54	***2.54***
8	2.69	2.48	3.41	***2.48***
9	2.52	***2.33***	2.91	2.91
10	2.49	2.29	2.87	***4.01***
11	1.29	***1.11***	2.49	0.00
12	2.53	3.50	2.63	***2.34***
13	2.74	***1.10***	3.84	1.10

Table 2.

Raw data for Experiment B. Alcohol drinking is given in g/kg. Data in bold/italics on test days were DMSO injection, while regular font on test days were SB injection. Drinking for non-test-days were average of 3 days of intake between the test days.

animal number	non-test-day drinking	test day 1	non-test-day drinking	test day 2	non-test-day drinking	test day 3	non-test-day drinking	test day 4
1	3.26	2.30	3.45	***3.36***	3.45	2.24	3.47	***4.93***
2	3.03	***2.42***	2.63	1.73	1.82	***1.73***	2.10	1.71
3	5.05	3.93	3.93	***2.15***	4.12	1.61	3.75	***2.14***
4	4.28	***1.75***	3.89	2.76	4.09	***2.76***	2.10	2.10
6	3.26	***3.09***	3.60	1.98	2.23	***2.48***	2.18	1.87
7	3.91	2.93	3.71	***2.18***	4.49	2.18	2.69	***1.62***
8	2.99	***1.79***	3.18	3.24	2.59	***3.24***	2.60	1.67
9	2.47	2.47	3.50	***2.41***	4.32	3.61	2.75	***2.35***
10	2.88	***0.00***	4.43	3.33	4.87	***3.33***	3.20	0.64
11	2.90	4.06	2.90	***0.54***	3.09	2.17	1.96	***1.60***
12	2.73	***2.34***	3.50	1.70	2.53	***1.13***	2.42	0.56