Immunophyllin Ligands Show Differential Effects on Alcohol Self-Administration in C57BL Mice

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ABSTRACT

High abstinence rates characterize alcohol-dependent liver graft recipients. The immunosuppressants cyclosporine A (CsA) and tacrolimus (TRL) also inhibit calcineurin (CLN) in the brain. Previously, we found that CsA reduces alcohol consumption in C57BL/6J mice. The goals of the present study were: 1) to compare the ethanol preference effects of CsA against TRL, as well as sirolimus (SRL), an immunosuppressant without CLN inhibition and 2) to establish that reduction of alcohol consumption is not caused by caloric reinforcement from these ligands. C57BL/6J mice trained to imbibe ethanol consumed ethanol or sucrose in a modified limited-access drinking-in-the-dark paradigm; test groups received vehicle or doses of CsA (5–50 mg/kg), TRL (0.5–2.5 mg/kg), or SRL (1.0–5.0 mg/kg) for 5 consecutive days, 30 min before each 2-h limited-access session. Brain CsA, TRL, and SRL concentrations were measured. CsA (p < 0.001) and TRL (p < 0.01) each decreased ethanol consumption, whereas SRL showed no significant effects at any dose. Effective doses included CsA at 10 mg/kg and above and TRL at 2.5 mg/kg. CsA (50 mg/kg) did not reduce sucrose consumption. Both CsA and TRL reached significant brain concentrations compared with very low values of SRL. These data suggest that CsA and TRL may reduce alcohol preference through central CLN inhibition rather than by immunosuppression.

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ABBRévIATIONS: AD, alcohol-dependent; CsA, cyclosporine A; TRL, tacrolimus; SRL, sirolimus; CLN, calcineurin; FKBP, FK506 binding protein; mTOR, mammalian target of rapamycin; LC, liquid chromatography; MS, mass spectrometry; ANOVA, analysis of variance; CyD, cyclophillin D.
then treated with CsA (Beresford et al., 2005). This effect occurred independently of hydration status. That study did not address possible mechanisms by which CsA lowered ethanol preference; two possibilities were considered. First, CsA might be acting through its immunosuppressant effect, namely systemic inhibition of calcineurin (CLN) and cyclophilins, or second, it might lower ethanol preference through its actions in specific inhibition of CLN activity in the brain (Schreiber and Crabtree, 1992; Swanson et al., 1992). From this we hypothesized that a second CLN/FKBP inhibitor, tacrolimus (TRL) [(3S)-(1S*,2S*,3S*,4S*)]-4S*,5R*,6S*,9E,12R*,14R*,15S*,16R*,18S*,19S*,26aR*)]-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26α-hexadecahydro-5,19-dihydroxy-3-(2-(4-hydroxy-3-methoxy)cyclohexyl)-1-methylthienyl]-16,14-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-15,19-epoxy-3H-pyrido [2,1-c][1,4] oxazacyclotricosine-1,7,20,21(4H,23H)-tetrone monohydrate], would evidence similar effects on alcohol preference, whereas sirolimus (SRL) [(3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)]=10,12,13,14,21,22,23,24,25,26,27,32,33,34,34α-hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[1S,5R,4R]-4-hydroxy-3-methoxy)cyclohexyl]-1-methylthienyl]-10-21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido [2,1-c][1,4] oxazaclyclohentriacontine-1,5,11,25,28,29,39H,6H,31H-pentone], an immunosuppressant agent without effects on brain CLN but also an inhibitor of FKBP and mammalian target of rapamycin (mTOR) (Edwards and Wandless, 2007), would have no effect on ethanol preference. If true, the evidence would support a CLN inhibition action in the brain as affecting ethanol preference. Substantiation of this also implied significant dose-related CsA and TRL concentrations in the brain.

At the same time, however, although hydration status was not related to CsA effects on ethanol preference, we could not rule out the possibility that the CsA effect alters appetite or produces caloric reinforcement.

The present experiment therefore entailed two principal hypotheses: 1) CsA and TRL, but not SRL, would result in lowered ethanol preference in mice and 2) CsA exerted its effect on ethanol preference independent of the animals’ caloric intake needs. To test these we chose a limited-access, multiple-dose paradigm.

Materials and Methods

Materials. CsA, TRL, and SRL were purchased from LC Laboratories (Woburn, MA). Sucrose and 2-hydroxy-β-propyl-cyclodextrin were purchased from Sigma-Aldrich (St. Louis, MO). Alcohol (ethanol) was purchased from Pharmaco AAAPER (Brockfield, CT).

Animals and Animal Care. All experimental protocols were submitted to and approved by both the Veterans Administration’s and University of Colorado’s Institutional Animal Care and Use Committees. Adult male C57BL/6J mice (aged 9 weeks old), purchased from The Jackson Laboratory (Bar Harbor, ME), were housed two per group in clear, acrylic plastic cages and acclimated to a reverse light cycle (lights off at 10:00 AM and on at 10:00 PM) initially for 3 weeks and for the duration of the experiment.

Drinking-in-the-Dark Ethanol Consumption. A drinking-in-the-dark paradigm of alcohol consumption, reported to work well in mice (Rhodes et al., 2005), was used to train the mice to consume adequate amounts of alcohol (see Supplemental Material for a detailed description and exposure schedule.) To measure alcohol consumption, a 10% alcohol (v/v, ethanol in tap water) bottle was placed on each test cage that had one floor mat and housed one mouse at noon, 2 h after the onset of the dark cycle, a time point reflecting previous reports that indicated increased diurnal drinking and eating activities (Goldstein and Kakihama, 1977). In brief, the routine procedures consisted of the following: on each day of the experiment, regular water bottles were removed at the appropriate time, and the mice were weighed beginning at 11:30 AM. For the initiation of the limited-access procedure, each mouse was moved from its home cage to a test cage containing a single 15 ml centrifuge tube equipped with a metal sipper (Lixit Med Associates Inc., St. Albans, VT). On experimental day 0, the water bottle was removed at 2:00 PM, 22 h before presentation of the limited-access bottle on experimental day 1. On day 1, the mice were offered a bottle containing only water during the limited-access period to acclimate them to the procedure; the water bottle was offered to them for 2 h (noon to 2:00 PM) to ensure adequate hydration. Beginning on day 2, a 0.6% ethanol solution was available during the limited-access period, 30 min daily, beginning at noon in the testing cages; for the remaining 23.5 h, water was available at the home cage. This procedure continued until the mice drank a significant and stable quantity of 10% alcohol solution. After this, the amount of ethanol consumption was measured for the duration of 2 h. The home-cage water bottles were returned to each cage immediately after completion of the limited-access session on each day at 2:30 PM. Finally, on days 16 to 21, the mice received an intraperitoneal injection of vehicle (20% 2-hydroxy-propyl-β-cyclodextrin solution) or the test drug at 30 min before the drinking session. At this point the access period to the 10% ethanol solution was increased to 2 h.

CsA, TRL, and SRL. After a period of habituation to 10% alcohol solution, each mouse received vehicle once per day for 5 days to establish the basal levels of alcohol consumption; on the sixth day, the mice were given CsA in one of three doses (1, 3, or 5 mg/kg; n = 10 mice each). This procedure was repeated in three new subgroups (n = 10 each) for CsA at 10, 30, or 50 mg/kg 30 min before the alcohol-access period. The CsA doses were based on that used in our pilot study (50 mg/kg) with the goal of assessing possible effects at varying active drug doses. Reported data on mouse metabolic rates and CsA use occasioned the original choice (Matsumura, 1996; Shuto, 1998, 1999). Calculated equivalent doses resulted in three intraperitoneal doses of TRL at 0.5, 1.5, and 2.5 mg/kg and SRL at 1, 3, and 5 mg/kg. The vehicle or the corresponding drug dosage was adjusted so that each mouse received injections of 0.1 ml per 10 g of body weight; intraperitoneal administration occurred once daily for 10 consecutive days.

Brain Concentrations of Active Drug. Because CsA, a high-affinity P-glycoprotein substrate, has only limited penetration through the blood-brain barrier (Serkova et al., 2000, 2001), we assessed CsA, TRL, and SRL brain tissue levels by using an high-performance liquid chromatography-MS assay with automated online sample extraction (LC/LC-MS) as described previously (Serkova et al., 2000, 2001). Brain tissues (approximately 500 mg of wet weight) were thawed and weighed. Brain samples were homogenized with 11 ml of 1 M KH2PO4 buffer, pH 7.4 by using a Tissue-Tearor 398 homogenizer (BioSpec Products, Inc., Bartlesville, OK). For protein precipitation, 100 µl of methanol/1 M ζ-ZnSO4 (80/20 v/v) was added to each 100-µl brain sample. The internal standards (cyclosporin D, ascomycin, and 28:40-Diacetyl rapamycin for CsA, TRL, and SRL, respectively) were dissolved in methanol/0.1% formic acid 9/1 (v/v), resulting in a concentration of 1 µl, and added to the brain samples. After centrifugation, 100 µl of the supernatant was injected onto the extraction column. Samples were washed with a mobile phase of 40% methanol and 60% 0.1% formic acid supplemented with 1 µM sodium formate. The flow rate was 5 ml/min, and the temperature for the extraction column was set to 65°C. After 0.75 min, the switching valve was activated, and the analytes were eluted in the backflush mode from the extraction column onto an analytical column (flow rate 0.5 ml/min). The mobile phase consisted of 90% methanol and 10% 0.1% formic acid supplemented with 1 µM sodium formate. The mass spectrometer was run in the selected ion mode.
and positive ions \([\text{M}+\text{Na}]^+\) were recorded. For all matrices, the analytical recovery was \(>90\%\).

The mass spectrometer was focused on: 1) \(m/z = 1224\) (CsA) and 1238 (cyclosporin D, internal standard); 2) \(m/z = 826\) (TRL) and 815 (ascomycin, internal standard); and 3) \(m/z = 936\) (SRL) and 1020 (28-40-diacyl rapamycin, internal standard). All LC/LC-MS experiments were performed in the Anesthesiology Mass Spectrometry Core, University of Colorado Anschutz Medical Campus (Aurora, CO).

**Sucrose Consumption.** To avoid procedure bias in assessing the active drug and control effects on alcohol versus sucrose consumption, we used the same limited-access model with sucrose in place of alcohol. Same-aged adult male C57BL/6J mice (\(n = 12\)) underwent the same acclimation process. For the initiation of the limited-access procedure, each mouse was moved from its home cage to a test cage containing a single 15-ml centrifuge tube equipped with a metal sipper (Lixit Med Associates Inc.). Each mouse had free access to water at its home cage initially for 2.5 h/day and ultimately 13 h/day during this initial 7-day period, with the sucrose concentration at 5% (wt/v). After day 8, with sucrose concentration at 15% or more there was no further water restriction, and water restriction was never extreme.

On each day of the training, regular water bottles were removed according to schedule, and the mice were weighed beginning at 11:30 AM. Accordingly, on experimental day 0, the water bottle was removed at 2:00 PM, 22 h before presentation of the limited-access bottle on experimental day 1. On day 1, the mice were offered bottles containing only water during the limited-access period to acclimate them to the procedure with access for 2 h (noon to 2:00 PM) to ensure adequate hydration. Beginning on day 2, a low concentration of sucrose solution was available during the limited-access period for 30 min from this day on, beginning at noon. The concentration of sucrose was increased every other day until it reached 30%. The home-cage water bottles were returned to each cage at the completion of the limited-access session on each day at 2:30 PM. Finally, on days 16 to 21, the mice were given an intraperitoneal injection of vehicle \((n = 6)\) or CsA 50 mg/kg \((n = 6)\) per group with consumption of the 30% sucrose solution measured at the end of the access period.

## Results

**Cyclosporine Reduces Ethanol Consumption in C57BL/6J Mice.** Tables 1 and 2 show that CsA doses of 10, 30, and 50 mg/kg were effective in reducing ethanol consumption \((F_{5,24} = 61.423; p < 0.001; \text{one-way ANOVA})\), varying in magnitude from 59 to 73%. Figure 1 presents these data in a log transform format to allow comparison with the other magnitude from 59 to 73%. Figure 1 presents these data in a log transform format to allow comparison with the other magnitude from 59 to 73%. Figure 1 presents these data in a log transform format to allow comparison with the other magnitude from 59 to 73%

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Vehicle</th>
<th>With Drug Treatment</th>
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<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA</td>
<td>10</td>
<td>2.26 ± 0.10</td>
<td>0.91 ± 0.10***</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.49 ± 0.17</td>
<td>0.68 ± 0.07***</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.55 ± 0.16</td>
<td>0.74 ± 0.05***</td>
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<tr>
<td>TRL</td>
<td>0.5</td>
<td>1.16 ± 0.45</td>
<td>0.94 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.27 ± 0.16</td>
<td>0.96 ± 0.33*</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1.01 ± 0.17</td>
<td>0.58 ± 0.15**</td>
</tr>
<tr>
<td>SRL</td>
<td>1.0</td>
<td>0.81 ± 0.76</td>
<td>0.76 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.66 ± 0.20</td>
<td>0.50 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>0.73 ± 0.17</td>
<td>0.78 ± 0.20</td>
</tr>
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</table>

One-tail t test, equal variance: *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).

**CsA Does Not Decrease Sucrose Consumption.** To determine whether high doses of CsA could cause caloric reinforcement that might reduce alcohol consumption, we tested the effects of CsA (50 mg/kg) on consumption of 30% sucrose solution. The results show that mice consumed 5.47 ± 0.75 g/kg sucrose during the vehicle treatment period, and CsA (50 mg/kg) treatment increased, rather than decreased, the levels of consumption to 8.72 ± 0.95 g/kg \((t = 2.685; p < 0.03)\).

**Effect of Tacrolimus and Sirolimus on Alcohol Consumption.** The data in Tables 1 and 2 show that administration of TRL to mice significantly reduced ethanol consumption \((F_{5,27} = 4.782; p < 0.005; \text{one-way ANOVA})\) by a magnitude of 24 to 43%. However, post hoc Holm-Sidak analysis revealed that only the highest dose (2.5 mg/kg TRL) significantly reduced alcohol consumption \((p < 0.001)\), whereas both 0.5 and 1.5 mg/kg doses of TRL were not effective. Therefore, the estimated minimum effective dose for TRL was 2.5 mg/kg (see Supplemental Fig. 2S).

For SRL, the data show that none of the test doses \((1, 3, 5 \text{ mg/kg})\) reduced alcohol consumption significantly \((F_{5,24} = 1.523; p > 0.200; \text{one-way ANOVA})\) (Tables 1 and 2). Therefore, SRL seems to have no effect in reducing ethanol consumption (see Supplemental Fig. 3S).
Considered overall, the three animal batches showed different baseline rates of ethanol consumption (Tables 1 and 2). Analysis addressed this in treating each animal as its own control. To assess a dose-response relationship among these drugs in reducing alcohol consumption in C57BL/6J mice we estimated the EC50 for each agent. Figure 1 shows that estimate for CsA at approximately 10 mg/kg and TRL at approximately 5 mg/kg. SRL was not effective in the dose range we tested.

Brain CsA, TRL, and SRL Concentrations. Drug concentrations in the brain indicate both the ability of the drugs to enter the brain and their possible accumulation in brain tissue. Figure 2 shows a dose-dependent increase in the levels of CsA in brain tissues (F1,27 = 12.365; p < 0.001; one-way ANOVA). We only measured the effective dose of TRL and the largest dose of SRL. These results show that after injection of 2.5 mg/kg TRL the brain TRL was 16.9 ± 2.33 ng/g brain tissues. Only very low levels of SRL were detected. When we tried to correlate the effects of drug on alcohol consumption and the brain levels of the drug by using the minimum effective drug dose, we found that the ligands that inhibit ethanol consumption also have higher brain levels (Fig. 3).

Discussion

CsA Inhibits Alcohol Preference. The results of our study clearly show dose-dependent inhibition of alcohol consumption in the mice treated with CsA for 5 days. Moreover, we demonstrate that the CsA inhibitory effect on alcohol intake was probably not caused by caloric reinforcement. In a dose-dependent fashion CsA penetrated into the mouse brain to exert its inhibition of alcohol intake. These results replicate and extend our previous report on alcohol preference in CsA-treated mice that used a 24-h continuous-access paradigm (Beresford et al., 2005). In the present experiment, although the previous 50 mg/kg dose reduced alcohol preference, so did the lower doses of 30 and 10 mg/kg. Of interest, this effect seemed to wane at the 10 mg/kg dose after approximately 4 treatment days, whereas it continued at the two higher doses through the 5-day exposure period. Whether the effect becomes attenuated or lost at the higher doses must be addressed in experiments that entail a longer period of exposure. Both the observation of effect at a low dose and the possibility of attenuation have clear implications in possible human use of CsA or a similar agent in treating AD humans. Human studies must be done at different doses and with sufficient length of exposure to allow for the assessment of both possible effects.

At the same time, the results suggest that CsA’s effect on alcohol preference has little to do with alcohol’s caloric content as a reinforcement of alcohol use. CsA reduced alcohol preference but increased sucrose intake, rather than decreasing both as might be expected from caloric reinforcement. This suggests that the CsA effect on ethanol consumption seems specific to mechanisms that may target it specifically.

CsA, TRL, and SRL Implicate CLN Inhibition in Reducing Alcohol Intake. This study begins to address the mechanism of CsA action in reducing alcohol consumption in laboratory animals. Our results suggest two possible CsA effects: brain CLN inhibition and CLN-mediated immunosuppression.

With respect to the latter, CsA cyclic polypeptide blocks the activity of cyclophilin D (CyD), a peptidyl-prolyl cis-trans isomerase that catalyzes protein unfolding. The CsA/CyD complex binds to CLN and inhibits its phosphatase activity. Moreover, CLN inhibitors such as CsA and TRL are known to modify rewarding effects of alcohol through a DARPP-32 phosphorylation mechanism in dopaminergic neurons of ventral tegmental area-nucleus accumbens (Svenningsson et al., 2005). As an immunosuppressant, CsA has been postulated to block interleukin-2 transcription and translation as well as suppression of immune interferon-γ growth factor, resulting in a decrease in interleukin-1, leading to the blockade of lymphocyte activation (Almawi and Melemedjian, 2000; Maramattom and Wijdicks, 2004). Recent studies (Blednov et al., 2011) show that immune signaling may promote alcohol consumption, and it is possible that the administration of these immunosuppressants could suppress the abnormal brain immune signaling to reduce alcohol consumption. Whether the alcohol preference change found here relates to...
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its immune suppression effects remains to be determined, but the present data suggest that it is unlikely.

More likely, in our view, the antiethanol effects result from the inhibition of brain CLN itself that subsequently modulate neurotransmission networks, especially those mediated by glutamate and dopamine. The positive effects of CsA and TRL, and the lack of an effect in SRL’s case, suggest CLN inhibition over immunosuppression, because all three agents suppress immunity. Furthermore, TRL is approximately 10 times more potent than CsA as an immunosuppressant, whereas here its limiting effect on alcohol preference was much less than that for CsA, again suggesting brain CLN inhibition rather than immunosuppression may be the active mechanism. TRL binds to a different immunophilin, FKBP12, than does CsA. Like CsA/CyD, the TRL/FKBP complex acts peripherally to lessen the immune response by the inhibition of CLN (Kunz and Hall, 1993; Halloran, 1996). In the brain, however, the TRL/FKBP complex inhibits CLN’s phosphatase activity in a manner approximately similar to the CsA/CyD complex.

The actions of SRL, an analog of TRL, differ in important ways. SRL binds to the 12-kDa TRL-binding proteins, FKBP12, and additionally to mTOR. The mechanisms of action for TRL-FKBP12 and SRL-FKBP complexes, however, differ in several ways. SRL does not inhibit CLN but reduces mTOR activity (Hults et al., 1992; Koser et al., 1993; Sapers et al., 1995; Gummert et al., 1999a,b). This results in the inhibition of a later, interleukin-2-dependent step of T cell activations (Chen et al., 1995; Marx et al., 1995). What effect this might have on alcohol preference remains unknown, but we found no evidence for an effect in the present paradigm. Centrally, SRL does not permeate the brain nearly to the extent that CsA and TRL do, and, lacking CLN inhibitory properties, it would not be expected to result in N-methyl-D-aspartate or dopamine modulation.

In the present report, both CLN inhibitors reached significant concentrations in the brain and significantly reduced ethanol drinking in the test animals (Fig. 1). Each did so in a dose-dependent relationship with their brain concentrations (Fig. 2). The non-CLN inhibitor, SRL, did not show this same effect.

Limitations. With a view toward eventual human use in alcoholism treatment this study did not address two further considerations: location of action and side effects. Research in rodents suggests that the CNS striatum, including the caudate nucleus, putamen, nucleus accumbens, and the olfactory tubercle along with the hippocampus (Mitsushashi, 2000), contain the highest CLN activities in the brain. Whether these sites reflect the locales of CsA and TRL in limiting CLN actions remains to be seen. Given the importance of ventral tegmental area-nucleus accumbens dopaminergic transmission in modulating alcohol reward, further studies may target the location and mechanisms in the brain by which CLN inhibitors decrease alcohol intake as in our results.

One important side effect of both CsA and TRL is possible nephrotoxicity, and this toxicity seems to be related to high dosage (Calne et al., 1978; Paul, 2001). Clinically, CsA and TRL seem potentially nephrotoxic, whereas SRL does not seem to have this effect (Gummert et al., 1999a,b). Fortunately, the toxic side effects of CsA and TRL can be avoided with close monitoring of dose and discontinuation of the medication as renal function tests indicate. But the issue of possible nephrotoxicity and other potential side effects must be kept in mind in developing analogous CLN inhibitors as possible treatment agents for AD.

Conclusion. This study strongly suggests an antidiptic effect occasioned by the two CLN-inhibiting agents, CsA and TRL. Further research may bear out the implication that CLN inhibition can play a role in AD treatment in humans, a condition for which very few therapeutic agents have demonstrated clinical efficacy. For perspective we take note of a brief clinical report (Giles et al., 1990) that was published the same year as our clinical algorithm for evaluating liver transplant candidates. Those investigators mentioned that AD patients with liver transplants became mostly abstinent from alcohol while on immunosuppressants, but their observation failed to spark much interest in the alcohol research community. The view of those authors coincides with ours, however, and with the line of inquiry reported here. It is our hope that this avenue, beginning with observations in the clinic, leading to basic experiments such as those reported here, will circle back through an evidentiary path to inform and improve the treatment of AD persons.

Authorship Contributions

Participated in research design: Beresford, Serkova, and Wu.

Conducted experiments: Fay, Serkova, and Wu.

Performed data analysis: Serkova and Wu.

Wrote or contributed to the writing of the manuscript: Beresford, Serkova, and Wu.

References


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SUPPLEMENTAL FILES
METHOD

Limited-Access Drinking for C57BL mice: Adult Male C57BL/6J (C57BL) mice purchased from Jackson Laboratory will be used in these experiments. C57BL mice will be housed in 5 per group and acclimated to a reverse light cycle (lights off at 1000 and on 2200) for 14 days (the reversed light/dark cycle is implemented for convince of experiment and is found to work well in previous studies). After this period, the mice age 10-12 weeks will be housed individually in clear, acrylic plastic cages with metal grid floor for additional 7 day. After this period, the mice will be placed on a decreasing schedule of water restriction across a 7-day period designed to increase their motivation to drink ethanol during the restrict-access period. Table 4 details the fluid restriction and ethanol access schedule (Sharpe, Coste et al. 2005).

A modified version of this protocol was found to work well in C57BL mice (Finn, Belknap et al. 2005; Rhodes, Best et al. 2005).

Table 4. Schedule of water restriction, increase of ethanol (E) solution concentration, and access time for the study with limited access to sucrose drinking

<table>
<thead>
<tr>
<th></th>
<th>Water Deprivation</th>
<th>Access Solution</th>
<th>Time</th>
<th>Location</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>21.5 hr</td>
<td>Water</td>
<td>2 hr</td>
<td>Test Cage</td>
</tr>
<tr>
<td>Day 2</td>
<td>21.5 hr</td>
<td>0.6% E</td>
<td>30 min</td>
<td>Test Cage</td>
</tr>
<tr>
<td>Day 3</td>
<td>17 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>15 hr</td>
<td>1.2% E</td>
<td>30 min</td>
<td>Test Cage</td>
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<tr>
<td>Day 5</td>
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<td></td>
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<tr>
<td>Day 6</td>
<td>13 hr</td>
<td>2.5% E</td>
<td>30 min</td>
<td>Test Cage</td>
</tr>
<tr>
<td>Day 7</td>
<td>11 hr</td>
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The limited-access bottle will always be placed on the cage at 1200 hr, 2 hr after the onset of dark cycle. The time point was chosen on the basis of previous reports indicating an increased amount of drinking and eating activities at this time (Goldstein and Kakihana 1977). On each day of the experiment, regular water bottles will be removed at the appropriate time according to the schedule outlined in Table 4, and the mice will be weighed beginning at 1130 hr. Thus, on experimental day 0, the water bottle will be removed at 1400 hr, 22 hr before presentation of the limited-access bottle on experimental day-1. On day 1, the mice will be offered a bottle containing water only, with no ethanol, during the limited-access period, to acclimate them to the procedure and the water bottle will be offered to them for 2 hr (1200-1400) to ensure adequate hydration in these mice that are naïve to the test conditions. Beginning on day 2, a low concentration of ethanol solution will be available during the limited-access period for 30 min from this day on, beginning at 1200 hr. The concentration of ethanol will be increased every other day until it reaches 10%. The home cage water bottles will be returned to each cage 2 hr after completion of the limited-access session on each day at 1430 hr. Finally, to obtain data for higher levels of ethanol consumption during the limited-access period, on day 16-21, the mice will be given an i.p. injection of vehicle (saline) or test drug at 1000 hr, the access period to the 10% ethanol solution will be increased to 2 hr (1200-1400 hr) and water bottles will be returned to the home cage at 1600 hr.

Sucrose consumption: A modified method for measuring a limited access of alcohol preference in mice (Sharpe, Coste et al. 2005) will be adopted for this study, and the procedure will be the
same as the one we use for limited access of alcohol preference test proposed in this application. Therefore, we may not run into procedure bias for assessing the effects on alcohol preference and sucrose preference. Adult male C57BL mice, age 10-12 weeks old, will be individually housed in the home cage (clear polycarbonate cage with corncob lining) 3 weeks before the experiment. At the beginning of acclimating phase, the light cycle (lights off at 1000 and on at 2200) will be set to acclimate for 14 days. After this 14-day period, the mice will be placed on a decreasing schedule of water restriction across a 7 day period designed to increase their motivation to drink sucrose solution during the restrict-access period. For the initiation of the limited-access sucrose solution drinking, the mice will be moved from their home cage to a test cage in the test room distinct from the home room two hours before the onset of testing. The test cage contains a single 50 ml centrifuge tube equipped with a metal sipping mouth piece. The mice will have free access to water at their home cage initially for 2.5 h/day and ultimately 13 h/day during this 7-day period, and the maximal sucrose concentration will be 5% (wt/v). After this initiation period, beginning when 15% sucrose will be first offered, there will be no water restriction. Therefore, water restriction will never be extreme, and sucrose concentrations remained low to avoid excessive forced sucrose intake that might lead to aversion.

**Table 5. Schedule of water restriction, increase of sucrose (S) solution concentration, and access time for the study with limited access to sucrose drinking**

<table>
<thead>
<tr>
<th>Water</th>
<th>Access</th>
<th>Time</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deprivation</td>
<td>Solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>21.5 hr Water</td>
<td>2 hr</td>
<td>Test Cage</td>
</tr>
<tr>
<td>Day 2</td>
<td>21.5 hr 0.5% S</td>
<td>30 min</td>
<td>Test Cage</td>
</tr>
<tr>
<td>Day 3</td>
<td>17 hr 1.0% S</td>
<td>30 min</td>
<td>Test Cage</td>
</tr>
<tr>
<td>Day 4</td>
<td>15 hr 1.0% S</td>
<td>30 min</td>
<td>Test Cage</td>
</tr>
</tbody>
</table>
Day 5  13 hr   5.0% S  30 min  Test Cage  
Day 6  13 hr   5.0% S  30 min  Test Cage  
Day 7  11 hr   10.0% S  30 min  Test Cage  
Days 8-9 none  15.0% S  30 min  Test Cage  
Days 10-11 none   20.0% S  30 min  Test Cage  
Days 12-15 none  30.0% S  30 min  Test Cage  
Days 16-21 none  30.0% S  2 hr   Test Cage  

The limited-access bottle will always be placed on the cage at 1200 hr, 2 hr after the onset of dark cycle. The time point was chosen on the basis of previous reports indicating an increased amount of drinking and eating activities at this time (Goldstein and Kakihana 1977). On each day of the experiment, regular water bottles will be removed at the appropriate time according to the schedule outlined in Table 5, and the mice will be weighed beginning at 1130 hr. Thus, on experimental day 0, the water bottle will be removed at 1400 hr, 22 hr before presentation of the limited-access bottle on experimental day-1. On day 1, the mice will be offered a bottle containing water only, with no sucrose, during the limited-access period, to acclimate them to the procedure and the water bottle will be offered to them for 2 hr (1200-1400) to ensure adequate hydration in these mice that are naïve to the test conditions. Beginning on day 2, a low concentration of sucrose solution will be available during the limited-access period for 30 min from this day on, beginning at 1200 hr. The concentration of sucrose will be increased every other day until it reaches 30%. The home cage water bottles will be returned to each cage 2 hr after completion of the limited-access session on each day at 1430 hr. Finally, to obtain data for higher levels of sucrose consumption during the limited-access period, on day 16-21, the mice will be given an i.p. injection of vehicle (saline) or test drug at 1000 hr, the access period to the 30% sucrose solution will be increased to 2 hr (1200-1400 hr) and water bottles will be returned to the home cage at 1600 hr.
RESULTS

Effects of CsA on ethanol consumption: The mice were randomly grouped into three groups: CsA 10 mg/kg; CsA 30 mg/kg; or CsA 50 mg/kg. After training to drink a steady quantity of ethanol, these mice were given vehicle injections, once daily for 5 days. Data show that the vehicle did not significantly alter ethanol intake in these mice. On the 6th day, mice were given CsA 10 mg/kg, 30 mg/kg, or 50 mg/kg 30 min before ethanol drinking session. Figure 1S-A shows that all three doses of CsA were effective in reducing ethanol consumption. The averaged levels of ethanol consumption over 5 days of vehicle or CsA are shown in Figure 1S-B. All three groups drank about 2 – 2.5 g/kg ethanol during the vehicle treatments but significant decreases in ethanol intake compared to vehicle were recorded during CsA treatments [F(5,24)=61.42, p<0.001, one-way ANOVA].

Effects of TRL on ethanol consumption: The mice were randomly selected into three groups: TRL 0.5 mg/kg; TRL 1.5 mg/kg; or TRL 2.5 mg/kg. These mice were trained to consume a steady level of ethanol. These mice were given once daily of vehicle injection for 5 days then were followed by once daily injection of TRL 0.5 mg/kg, TRL 1.5 mg/kg, or TRL 2.5 mg/kg 30 min before ethanol consumption. Figure 2S-A shows the day-to-day levels of ethanol intake during vehicle or drug injections. Figure 2S-B shows that the mice drank on average about 1 - 1.3 g/kg ethanol during the vehicle treatment period. Analysis of the data shows no significantly different in ethanol intake by all three groups during vehicle treatment but only TRL 2.5 mg/kg shows significant decrease in ethanol consumption [F(5,27)=4.782, p<0.003, one-way ANOVA] with post-hoc Pairwise multiple comparison.

Effects of SRL on ethanol consumption: The mice were randomly selected into three groups: SRL 1.0 mg/kg; SRL 3.0 mg/kg; or SRL 5.0 mg/kg. These mice were trained to consume steady
levels of ethanol and were given once daily injection of vehicle or SRL. Results show that neither vehicle nor SRL was able to reduce ethanol consumption in these animals (Figure 3S).

REFERENCES


FIGURE LEGENDS FOR THE SUPPLEMENTARY FIGURES.

Figure 1S. CsA reduces ethanol consumption in C57BL mice.

The mice (n=30) were trained to consume steady levels of ethanol in DID paradigm. Following the training, the mice were given once daily injection of vehicle for 5 days, then CsA (10, 30, or 50 mg/kg) for another 5 days. The levels of ethanol consumption were recorded. A. Daily average of ethanol intake for each group of animals. B. The overall average of ethanol consumption during 5 days of vehicle and CsA treatments. *** p<0.001.

Figure 2S. TRL reduces ethanol consumption in C57BL mice.

A new batch of C57BL mice (n=30) were trained to consume steady levels of ethanol. These animals were given a daily injection of vehicle for 5 days and were followed by once daily injection of test TRL for another 5 days. The effects of vehicle or TRL (0.5, 1.5, or 2.5 mg/kg) on ethanol consumption are expressed. A. Daily average of the level of ethanol consumption during vehicle and TRL treatment periods. B. Five-day averages of ethanol consumption in vehicle or TRL treatment period. ** p<0.003.

Figure 3S. SRL fails to reduce ethanol consumption in C57BL mice.

Another batch of C57BL mice (n=30) were purchased and trained to consume a steady level of ethanol in a DID drinking paradigm. Following the training, mice were given a daily injection of vehicle for 5 days and then once daily injection of SRL (1.0, 3.0, or 5.0 mg/kg). Ethanol consumption of each mouse during these periods was recorded. A. Daily average of ethanol consumption for each group of mice. B. Five-day average of ethanol consumption for each group of mice during the vehicle or SRL treatment period.
Figure 3 S

(A) Graph showing ethanol intake (g/kg) over treatment days for different SRL doses: SRL 1.0 mg/kg (filled circles), SRL 3.0 mg/kg (open circles), and SRL 5.0 mg/kg (filled triangles). The graph also indicates the vehicle (dashed line) and SRL treatment (solid line) periods.

(B) Bar graph displaying ethanol intake (g/kg) for different conditions: V1, SRL1, V2, SRL3, V3, SRL5, with error bars indicating variability.