Neurokinin 1 Receptor Antagonism as a Possible Therapy for Alcoholism

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Supporting Online Material

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Neurokinin 1 Receptor Antagonism as a Possible Therapy for Alcoholism

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Alcohol dependence is a major public health challenge in need of new treatments. As alcoholism evolves, stress systems in the brain play an increasing role in motivating continued alcohol use and relapse. We investigated the role of the neurokinin 1 receptor (NK1R), a mediator of behavioral stress responses, in alcohol dependence and treatment. In preclinical studies, mice genetically deficient in NK1R showed a marked decrease in voluntary alcohol consumption and had an increased sensitivity to the sedative effects of alcohol. In a randomized controlled experimental study, we treated recently detoxified alcoholic inpatients with an NK1R antagonist (LY686017; $n = 25$) or placebo ($n = 25$). LY686017 suppressed spontaneous alcohol cravings, improved overall well-being, blunted cravings induced by a challenge procedure, and attenuated concomitant cortisol responses. Brain functional magnetic resonance imaging responses to affective stimuli likewise suggested beneficial LY686017 effects. Thus, as assessed by these surrogate markers of efficacy, NK1R antagonism warrants further investigation as a treatment in alcoholism.

Alcohol use accounts for 4% of global disease burden (*1*). Alcohol dependence, or alcoholism, is characterized by a chronic relapsing course, in which alcohol-associated cues and stress are known relapse triggers (*2*–*6*). Recent research suggests that neural systems mediating behavioral stress responses may offer useful targets for pharmacotherapy of alcoholism. In animal models, excessive alcohol consumption that results from a history of alcohol dependence is accompanied by increased behavioral sensitivity to stress (*7*). Up-regulated corticotropin-releasing hormone (CRH) signaling in extrahypothalamic brain sites contributes to these dependence-induced changes, but other stress-related neurotransmitters may also play a role.

One such neurotransmitter is substance P (SP), which together with its preferred neurokinin 1 receptor (NK1R) is highly expressed in brain areas involved in stress responses and drug reward, including the hypothalamus, amygdala, and nucleus accumbens. In rodents, psychological stressors induce release of SP in the amygdala, whereas genetic deletion or pharmacological blockade of NK1R inhibits the associated behavioral responses (*8*). Furthermore, genetic deletion of NK1Rs causes a loss of conditioned place preference for opiates and opiate self-administration (*9*, *10*). In humans, the NK1 antagonist GR205171 reduces symptoms of social anxiety and suppresses brain responses to the Trier Social Stress Test (TSST) (*11*). Together, these findings suggest that blockade of NK1Rs might modulate stress- and reward-related processes of importance for excessive alcohol use and relapse. To our knowledge, no data are presently available to address this hypothesis.

We first explored preclinically whether inactivation of NK1R might modulate stress- and reward-related processes that impact alcohol use. We chose a genetic inactivation strategy, because available NK1R antagonists have limited activity in rats and mice, because of insufficient NK1R amino acid homology between humans and these rodent species (*8*). We evaluated NK1R null-mutant mice for voluntary alcohol consumption, alcohol sensitivity, and alcohol metabolism (*12*). NK1R null mice (*13*) were back-crossed into a C57BL/6 background for 10 generations to ensure that there was adequate voluntary alcohol consumption in control animals (*14*). We used a two-bottle free-choice model with increasing alcohol concentration, and alcohol was continuously available. Wild-type littermates (+/+) ultimately consumed in excess of 10 g alcohol/kg of body weight per day at the end of an escalation procedure in which alcohol concentration was gradually increased from 3 to 15% over 60 days.

Alcohol consumption by NK1R$^{-/-}$ mice was markedly lower than that by wild-type controls (Fig. 1A). The difference was most prominent at higher alcohol concentrations, at which consumption motivated by pharmacological alcohol effects dominates over intake for taste, calories, or other nonpharmacological effects (*14*). Alcohol consumption by heterozygous (+/–) mice was similar to wild-type controls, highlighting the necessity for near-complete inactivation of NK1Rs to suppress alcohol consumption. Sev-

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eral observations indicated that the reduction in alcohol intake was specific. Thus, relative preference for alcohol was also markedly reduced [($F_{2,45}$ = 13.6, P < 0.0001), whereas water intake was unaffected by genotype ($F_{2,45}$ = 0.54, P = 0.71)]. Additional control experiments indicated that the reduction in alcohol intake was not caused by altered thirst or taste preference (*12*).

Alcohol sensitivity, measured as the time required to regain the righting reflex after a high dose of alcohol (3.5 g/kg), was markedly increased in NK1$^{-/-}$ mice (Fig. 1B). This profile is consistent with an abundance of animal data showing an inverse relation between alcohol sensitivity and motivation to consume alcohol, as well as human findings of an inverse relation between alcohol sensitivity and alcoholism risk (*15*).

To assess the potential clinical relevance of these results, we conducted an experimental study in human alcohol-dependent patients (fig. S1 and table S1), in which we evaluated the effects of NK1R antagonism on processes related to relapse. We studied LY686017 (*16*), a high-affinity, selective NK1R antagonist that is orally available and brain penetrant (Fig. 2A). Preclinical pharmacology, safety, and human pharmacokinetics of LY686017 will be reported separately. Activity of LY686017 to reduce drinking was not assessed in preclinical experiments because, similar to other human NK1R antagonists, LY686017 has insufficient affinity for the mouse or rat NK1R. LY686017's brain penetrance and NK1R occupancy were established in a human positron emission tomography (PET) study of eight healthy volunteers (*12*). On the basis of these results, we used a dose of 50 mg daily, which yields a >90% blockade of central NK1R. LY686017 at this dose was well tolerated (table S2) (*12*), in agreement with prior reports indicating that NK1 antagonists as a class are safe and well tolerated (*8*).

Given the role of SP and NK1 receptors in stress and anxiety responses, we targeted subjects with high trait anxiety. Participants (25 per arm; age: 21 to 65 years) had a diagnosis of alcohol dependence, alcohol problems as the primary complaint, alcohol use within the last month, and >39 on the Spielberger Trait Anxiety Inventory (STAI) (*17*). They were hospitalized throughout the study and had completed withdrawal treatment before entering the study if needed. For details, see (*12*).

We found that LY686017 suppressed spontaneous alcohol cravings, as measured by the Alcohol Urge Questionnaire (AUQ) (Fig. 2B). As expected, these cravings declined over time in the protected inpatient environment and were minimal in the majority of patients by the end of the 4-week study period. However, overall, the cravings were sufficiently intense to allow detection of the medication effect. Weekly ratings by a blinded observer likewise suggested that LY686017 had a beneficial effect on global improvement and severity measured by the Clinician's Global Impression (CGI) scale (Fig. 2B). In contrast, twice-weekly ratings collected to obtain measures of general psychopathology showed no treatment effects on anxious or depressive psychopathology ($F_{1,102}$ = 0.7, P = 0.40). This suggests that the improvements observed might be specific for brain processes related to alcoholism.

We further measured craving responses to a combined stress (TSST) and alcohol-cue challenge (*12*) and found that treatment with LY686017 reduced the resulting AUQ craving response (Fig. 3A). Treatment with LY686017 also suppressed the concomitant cortisol response to the challenge (Fig. 3B). A study using PET has previously shown that GR205171, another NK1 antagonist, suppresses amygdala activation in response to the TSST in social phobics, but in that study, NK1R antagonism failed to produce effects on subjective, self-reported measures in response to the challenge (*11*). This shows that subjective responses to a challenge are transient, and their detection is critically dependent on the time point and the assessment instrument chosen. The fact that we detected consistent effects of LY686017 on both subjective and neuroendocrine responses to the craving challenge, therefore, supports the robustness of the LY686017 effect.

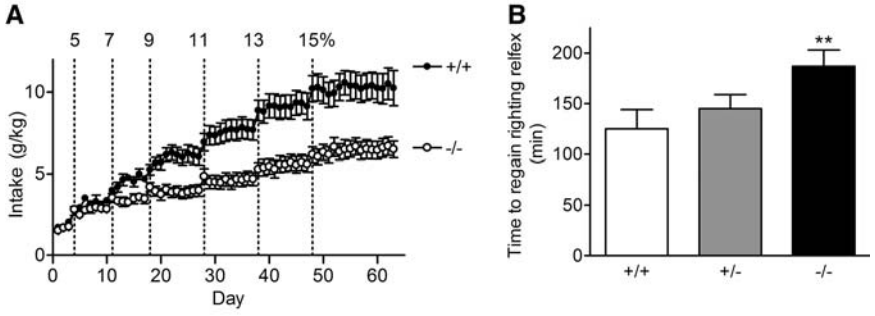

Fig. 1. Voluntary alcohol intake and alcohol sensitivity in NK1 null (–/–), heterozygous (+/–) and wild-type (+/+) mice. (**A**) Voluntary consumption in a two-bottle free-choice paradigm with continuous alcohol access. Alcohol was introduced as a 3% solution in water, and concentration was escalated over time, as indicated above the graph, to avoid taste aversion and to achieve pharmacologically active levels of alcohol consumption. The other bottle contained water. Data are means ± SEM; n = 16 per group. The +/– group is omitted for clarity; its alcohol consumption was virtually identical to that of +/+ mice. There was a main genotype effect ($F_{2,40}$ = 929.6, P < 0.00001). On Tukey's post hoc test, –/– mice differed from both +/+ and +/– mice (both: P < 0.001); the latter two genotypes were virtually identical (P = 0.92). (**B**) Alcohol sensitivity in mice, measured as the time to regain the righting reflex after a 3.5-g/kg dose of alcohol. Data are means ± SEM; n = 12 to 17/group). Time to regain the righting reflex was significantly longer for the NK1$^{-/-}$ mice as compared with their wild-type controls ($F_{2,42}$ = 7.8, P = 0.0014; Tukey's post hoc P = 0.001 –/– vs. +/+).

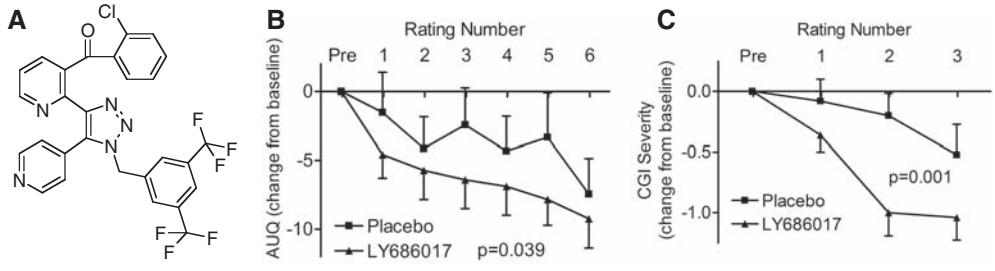

Fig. 2. Effect of the NK1R antagonist LY686017 on spontaneous cravings and global well-being in hospitalized alcoholics. (**A**) LY686017 (2-chloro-phenyl)-{2-[5-pyridin-4-yl-1-(3,5-bistrifluoromethyl-benzyl)-1H-[1,2,3]triazol-4-yl]-pyridin-3-yl}-methanone) (*16*), the selective, brain-penetrant NK1R antagonist used in this study. (**B**) Change from baseline for spontaneous alcohol cravings, as measured by twice-weekly ratings using the AUQ. The first value reflects the baseline rating obtained during the placebo lead-in week; the following ratings are from the active treatment phase. Data are means ± SEM. Baseline values for placebo and LY686017 groups were 19.1 ± 2.4 and 19.7 ± 2.3, respectively. Controlling for pretreatment baseline and sex, there was a highly significant decline of cravings over time ($F_{5,220}$ = 37.05, P < 0.0001), and a significant main effect of treatment ($F_{1,100}$ = 4.4, P = 0.039) on this outcome. (**C**) Change from baseline on weekly observer-based ratings using the Severity scale of the CGI rating questionnaire. The first value reflects the baseline rating obtained during the placebo lead-in week; the following ratings are from the active phase. Average baseline ratings for the placebo and LY686017 groups were 3.80 ± 0.15 and 3.88 ± 0.13, respectively. Data are means ± SEM. Controlling for pretreatment baseline, sex, and body mass index, there was a significant effect of treatment ($F_{1,42}$ = 11.9, P = 0.001). Very similar results were obtained on the improvement scale of the CGI (main treatment effect: $F_{1,44}$ = 8.4, P = 0.006).

Finally, we investigated whether LY686017 affected brain responses to standardized affective stimuli, with a pattern suggestive of beneficial effects on relapse-related mechanisms. We measured blood oxygen–level–dependent (BOLD) activity using functional magnetic resonance imaging (fMRI), following presentation of negative and positive emotional stimuli from the International Affective Picture System (IAPS) (*18*) and pictures of alcoholic or neutral beverages (*12*). Alcoholics exhibit exaggerated behavioral and brain responses to images associated with negative affect, and conversely, exhibit reduced brain responses to standard positive images (*19*). In agreement with these earlier observations, we found that placebo-treated alcoholics showed robust brain responses to negative affective images. Alcoholics who received LY686017 had less activation to the negative images than the placebo group in several brain regions associated with emotional response to visual stimuli [Fig. 4 and table S3 (*12*)]. In particular, the LY686017 group had less activation in the insula, a brain region whose activation correlates with subjective measures of craving (*20*), and which has recently been implicated in the maintenance of addictive behavior (*21*). In addition, the LY686017-treated group showed greater brain activation to the positive IAPS images than the placebo-treated group (Fig. 4). This may reflect an overall shift in the balance between positive and negative emotionality as indicated by the improvement detected on the CGI. A recent report suggests that greater activation to positive images in the striatum and thalamus of treated alcoholics predicts less alcohol consumption over the next 6 months (*22*).

Basic neuroscience research has identified numerous candidate targets for pharmacological treatment of alcoholism (*4*), but translation into clinical development has been limited. Surrogate markers of efficacy that can be evaluated in alcoholics under safe, closely monitored conditions can facilitate translation. In this context, self-reported cravings are of interest, because they are triggered in humans by the same types of stimuli that, in animal models, induce relapse to alcohol-seeking (*3*, *5*, *6*). Human data also show that cravings correlate with clinical outcomes (*23*) and are sensitive to a clinically effective alcoholism medication, naltrexone (*24*). For these reasons, we chose spontaneous as well as challenge-induced cravings as primary outcomes in our study. Both these outcomes were beneficially affected by the NK1R antagonist.

The concept of craving and its ability to predict clinical outcomes has also invited some debate (*25*). Although effects on cravings and clinical outcomes do correlate in the case of naltrexone, it is unknown whether this relation will hold up for other pharmacodynamic mechanisms. This will only be possible to establish once additional compounds with clinical efficacy are identified. Until then, a translational approach that is guided by animal data and combines craving measures with a profile across a broader range of experimental outcomes appears to offer an attractive approach to drug development for alcoholism. Using this approach, we provide here consistent data across a range of measures suggesting that NK1R antagonism might be of therapeutic value in alcoholism. A possible mechanism for this is suppression of pathologically elevated amygdala activity thought to develop following a history of alcohol dependence (*7*). Our results were obtained in anxious alcoholics. Larger trials, stratified for anxiety measures, will be required to address whether the effect of NK1R antagonism is specific for this population.

Other NK1R antagonists have previously been tested clinically as a therapy for major depression (*8*, *26*), but the results were inconsistent, and development of these drugs was stopped. Additional studies will be required to determine whether development of NK1R antagonists for alcoholism will be more successful, but recent findings with another stress-related neuropeptide system, CRH, suggest that this may be the case. Thus, analysis in animal models of alcoholism shows that the CRH system is quiescent under physiological or near-physiological conditions, and under these conditions, no activity of CRH antagonists is found. The CRH system is, however, pathologically activated following a history of alcohol dependence, revealing activity of CRH antagonists (*7*). These findings are in agreement with a general principle proposed for neuropep-

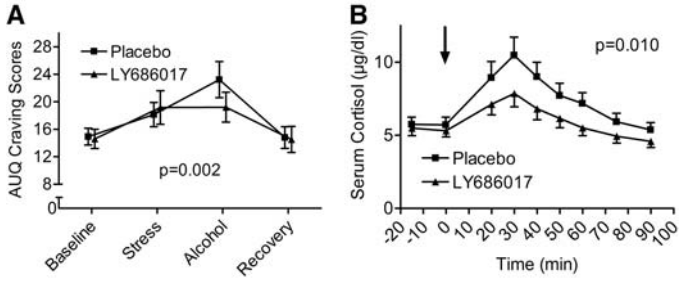

Fig. 3. Effect of the NK1R antagonist LY686017 on subjective craving and neuroendocrine response to a challenge session in hospitalized alcoholics. (**A**) Subjective craving ratings on the AUQ. Data are means ± SEM. Possible score range is 8-56. Controlling for sex and age, there was a robust craving response to the challenge on the AUQ, as shown by an effect of the repeated measures (time) factor ($F_{3,129} = 4.8$; $P = 0.003$), and a highly significant post hoc difference of both the poststress and the post–alcohol cue value versus baseline (Dunnett's test, $P < 0.001$ for each comparison); the recovery rating was back to baseline level. A modulation of the challenge response by treatment was shown as a time × treatment interaction ($F_{3,129} = 5.22$; $P = 0.002$). Responses were also obtained on a simple visual analog scale (VAS). Although this was less sensitive, a similar effect was suggested at a trend level (time × treatment: $F_{4,176} = 2.10$; $P = 0.08$). There was also a robust anxiety response ($F_{3,132} = 4.1$, $P = 0.008$), but no treatment or interaction effect on this variable. (**B**) Neuroendocrine response. Data are means ± SEM; arrow points to start of challenge. Controlling for sex, there was a robust cortisol response to the combined stress and cue exposure, as shown by a highly significant effect of the repeated measures (time) factor ($F_{8,344} = 12.9$, $P << 0.0001$). A significant differential response to the combined stress and cue exposure as a function of treatment was demonstrated by the interaction of time × treatment ($F_{8,344} = 2.6$, $P = 0.010$).

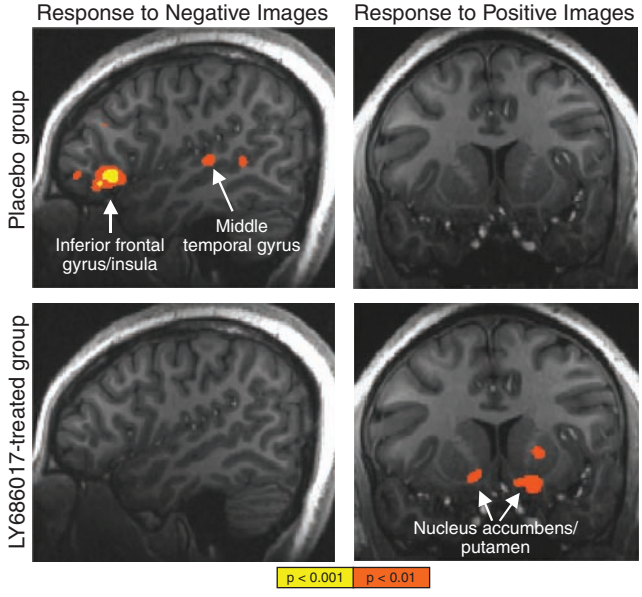

Fig. 4. Effects of LY686017 on fMRI BOLD responses to visual affective stimuli in hospitalized alcoholics. In the placebo group, there were robust activations to the negative stimuli in the inferior frontal gyrus, insula, and middle temporal gyrus; the LY686017-treated group had significantly less activation in these areas. The placebo group had very little activation in response to the positive emotional stimuli; the LY686017-treated group had greater activation in the thalamus, caudate (including ventral putamen), lingual gyrus, and several temporal areas. Group statistical maps are superimposed upon a T1 structural image in Talairach space. See table S2 (*12*) for details.

tide systems (*27*). Along these lines, activation of the SP-NK1R system may not be a consistent feature of depressive illness. If, however, a pathological activation of the SP-NK1R system follows a history of alcohol dependence, similar to CRH, NK1 antagonism may have a considerable potential as a treatment for alcoholism.

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References

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Using Engineered Scaffold Interactions to Reshape MAP Kinase Pathway Signaling Dynamics

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Scaffold proteins link signaling molecules into linear pathways by physically assembling them into complexes. Scaffolds may also have a higher-order role as signal-processing hubs, serving as the target of feedback loops that optimize signaling amplitude and timing. We demonstrate that the Ste5 scaffold protein can be used as a platform to systematically reshape output of the yeast mating MAP kinase pathway. We constructed synthetic positive- and negative-feedback loops by dynamically regulating recruitment of pathway modulators to an artificial binding site on Ste5. These engineered circuits yielded diverse behaviors: ultrasensitive dose response, accelerated or delayed response times, and tunable adaptation. Protein scaffolds provide a flexible platform for reprogramming cellular responses and could be exploited to engineer cells with novel therapeutic and biotechnological functions.

In cells, signaling proteins that make up a pathway are often physically organized into complexes by scaffold proteins (*1*–*3*). Scaffolds direct information flow; they promote signaling between proper protein partners and prevent improper cross talk. Scaffolds may also play a role in shaping the quantitative response behavior of a pathway. The scaffold complex could serve as a central hub for feedback loops that modulate the recruitment or activity of pathway members on the scaffold. Such feedback loops could tune pathway dose response and dynamics—the change in output over time. Quantitative response behavior is critical for signaling; the behavior of a pathway must match its specific physiological function (*4*). Scaffolds may therefore provide a platform for evolutionarily tuning response behaviors for optimal fitness (*5*, *6*).

We used a synthetic biology approach to explore this hypothesis; we tested whether a scaffold protein can be used as a platform for engineering synthetic feedback loops and whether these loops can be used to systematically reshape pathway response behavior (*7*). We used the yeast mating mitogen-activated protein (MAP) kinase pathway as a model system, because it is highly tractable for pathway engineering. First, proper connectivity of this pathway is dependent on the scaffold protein Ste5, which binds the three core kinases—Ste11 (a MAP kinase kinase kinase or MAPKKK), Ste7 (a MAP kinase kinase, or MAPKK), and Fus3 (a MAP kinase, or MAPK)—that successively phosphorylate and activate one another (Fig. 1A) (*8*, *9*). The critical role in determining pathway connectivity is highlighted by the observation that chimeric scaffolds can be used to redirect pathway input and output linkages (*10*, *11*). Second, MAP kinase pathways appear to be functionally plastic; they are found in all eukaryotic species, but in individual cases display widely varied behaviors. For example, the yeast mating pathway shows a largely linear transcriptional response (*12*–*14*), whereas the *Xenopus* oocyte maturation pathway displays a switchlike dose response (*15*). MAPK pathways also show diverse dynamic behavior; some yield a sustained response to stimulation, whereas others show a pulselike transient response. These distinct pathway dynamics are critical for determining physiological output (*16*–*21*).

Our goal was to overlay the endogenous mating pathway with synthetic feedback loops in order to systematically alter its response to mating pheromone (α-factor) stimulation. A simple way to construct a synthetic feedback loop would be to dynamically recruit pathway modulators to the scaffold in a manner that is dependent on pathway output. We first tested whether constitutive recruitment of modulator proteins could alter pathway flux. We created a new recruitment site on Ste5 by fusing a leucine zipper heterodimerzation module (*22*) to its C terminus. Modulator proteins fused to complementary zippers were expressed and recruited to the scaffold (Fig. 1B). Two pathway modulators were recruited:

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