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A Combination of Naltrexone + Varenicline Retards the Expression of a Genetic Predisposition Toward High Alcohol Drinking

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Abstract

Background—This study examined whether naltrexone (NTX) or varenicline (VAR), alone or in combination, can retard the phenotypic expression of a genetic predisposition toward high alcohol drinking in rats selectively bred for high alcohol intake when drug treatment is initiated prior to, or concomitantly with, the onset of alcohol drinking.

Methods—Alcohol-naïve P rats were treated daily with NTX (15.0 mg/kg BW), VAR (1.0 mg/kg BW), a combination of NTX (15.0 mg/kg BW) + VAR (1.0 mg/kg BW), or vehicle (VEH) for 2 weeks prior to, or concomitantly with, their first opportunity to drink alcohol and throughout 21 days of daily 2-hour alcohol access. Drug treatment was then discontinued for 3 weeks followed by reinstatement of drug treatment for an additional 3 weeks.

Results—When P rats were pretreated with drug for 2 weeks prior to onset of alcohol access, only NTX+VAR in combination blocked the acquisition of alcohol drinking in alcohol-naïve P rats. When drug treatment was initiated concomitantly with the first opportunity to drink alcohol, NTX alone, VAR alone, and NTX+VAR blocked the acquisition of alcohol drinking. Following termination of drug treatment, NTX+VAR and VAR alone continued to reduce alcohol drinking but by the end of 3 weeks without drug treatment, alcohol intake in all groups was comparable to that seen in the vehicle-treated group as the expression of a genetic predisposition toward high alcohol drinking emerged in the drug-free P rats. After 3 weeks without drug treatment, reinstatement of NTX+VAR treatment again reduced alcohol intake.

Conclusion—A combination of NTX+VAR, when administered prior to, or concomitantly with, the first opportunity to drink alcohol, blocks the acquisition of alcohol drinking during both initial access to alcohol and during a later period of alcohol access in P rats with a genetic predisposition toward high alcohol intake. The results suggest that NTX+VAR may be effective in curtailing alcohol drinking in individuals at high genetic risk for developing alcoholism.

Keywords

Alcohol Drinking; P rats; Naltrexone; Varenicline

Alcoholism increases the lifetime risk of physical illness, criminal and domestic violence, and financial and social insecurity, (Fergusson et al., 2013) and represents a large economic burden on the U.S. healthcare system (\$24.5B in 2006) (Bouchery et al., 2011). Both psychosocial and pharmacologic approaches are being used to treat alcoholism once it develops, but a major goal continues to be the prevention of heavy drinking that leads to the development of alcohol use disorder (AUD) (American Psychiatric Association, 2013). A foolproof way to prevent AUD is to never start drinking in the first place but, given the legal availability and popularity of alcohol, combined with a societal tolerance toward drinking, a more realistic goal may be to reduce, rather than eliminate, alcohol drinking (White and Hingson, 2014) which can prevent the most damaging health consequences of drinking that often occur after prolonged and extensive consumption.

As early as the 1980s it was recognized that the probability of drinking heavily is, in part, genetically determined (Cadoret et al., 1980; Cloninger et al., 1981) as evidenced by the fact that the incidence of alcoholism is higher in families of alcoholics than in the general population. For instance, sons of alcoholics were found to be approximately 3 to 5 times more likely to become alcoholic than were sons of nonalcoholics (Cotton, 1979; Goodwin et al., 1974). This increased risk could not be attributed to environmental factors only, such as availability of alcohol in the home or mimicking the behavior of drinking adults, since sons of alcoholics who were adopted by nondrinking families in early life were still 3 times more likely to become alcoholic than were similarly adopted sons of nonalcoholics (Cadoret et al., 1980; Cloninger et al., 1981; Goodwin et al., 1973, 1974). A study of the age-corrected lifetime prevalence of alcohol dependence in over 5,000 relatives of 1269 probands with alcohol dependence found that 45% of alcohol dependent subjects had one or both parents who were alcohol dependent themselves (Nurnberger et al., 2005). A current popular approach for exploring the genetics of alcoholism is the use of genotyping to identify genes related to genetic risk for alcohol abuse and dependence (Bierut et al., 2012; Edenberg and Faroud, 2013). Rodents selectively bred for high voluntary alcohol drinking, such as rats of the alcohol-preferring (P) line, are a good model of individuals at high genetic risk for the development of alcoholism. These rat lines are uniquely suited for identifying pharmacotherapeutic agents that have the potential to inhibit the expression of a genetic predisposition toward high alcohol drinking.

Naltrexone (NTX), a classic opioid receptor antagonist, is the most extensively characterized and effective agent currently used to treat alcohol abuse and alcoholism (for review see Froehlich et al., 2003; O'Malley and Froehlich, 2003). NTX decreases alcohol drinking in rodents in a variety of situations including during unrestricted access to alcohol, during restricted or scheduled access to alcohol, and during reinstatement of access to alcohol following alcohol deprivation. In humans, NTX decreases heavy drinking in both alcohol-dependent and nondependent alcohol drinkers (for review see Chick et al., 2000; Hendershot et al., 2016). NTX also reduces alcohol craving in individuals with AUD (Volpicelli et al.,

1992) and reduces alcohol drinking in young adults (O'Malley et al, 2015). However, not all studies have found significant effects of naltrexone in the treatment of AUD (Krystal et al., 2001). Recently varenicline (VAR) has also been shown to reduced alcohol drinking in rodents (Froehlich et al., 2016; Steensland et al., 2007) and humans (Falk et al., 2015; Fucito et al., 2011; Litten et al., 2013; McKee et al., 2009). VAR, which is marketed as CHANTIX® in the United States, and as CHAMPIX in Europe and elsewhere, is an orally active $\alpha 4\beta 2$ nicotinic acetylcholinergic receptor (nAChR) partial agonist that was FDA approved for smoking cessation in 2006 (Rollema et al., 2007). VAR reduces alcohol consumption in both alcohol-dependent (Litten et al., 2013) and nondependent (Mitchell et al., 2012) individuals and in both smokers and nonsmokers (Falk et al., 2015; Litten et al., 2013). However, a reduction in alcohol consumption is not always seen (Schacht et al., 2014). Both NTX alone and VAR alone can reduce alcohol craving (Fucito et al., 2011; O'Malley et al., 1996) and the subjective reinforcing effects of alcohol ("high" or "intoxication") (McKee et al., 2009; Volpicelli, 1995). A combination of NTX+VAR has been reported to decrease both smoking (Ray et al., 2015; Roche et al., 2015) and alcohol drinking (Ray et al., 2014). In the current study, we hypothesized that combining NTX with VAR would block the expression of a genetic predisposition toward high alcohol drinking if the combination was given either prior to, or concomitantly with, the first opportunity to drink alcohol. To test this hypothesis in humans would require that the combination be administered prior to, or beginning with, the first alcohol drinking experience which, in individuals at high genetic risk for alcoholism, can be at an early age (Kuperman et al., 2013). Initiating a pharmacological intervention at an early age in humans is not likely to be undertaken unless evidence exists that it has a potential long-term benefit. The current study investigated whether NTX or VAR alone, or in combination, when administered prior to, or concomitantly with, the first opportunity to drink alcohol, can prevent the expression of a genetic predisposition toward high alcohol drinking in alcohol-naïve P rats that have been selectively bred for alcohol preference and high alcohol drinking.

MATERIALS AND METHODS

Subjects

Genetic animal models have been particularly useful in furthering our understanding of a number of human diseases that are, in part, genetically determined, including alcoholism. In 1974, Drs. Li and Lumeng began a selective breeding program for high and low alcohol drinking in rats which resulted in the derivation of two rat lines: the alcohol-preferring (P) and nonpreferring (NP) lines (Li et al., 1979) that differed widely in voluntary alcohol intake. Rats of the P line, which voluntarily consume large quantities of alcohol, have been extensively characterized both behaviorally and physiologically (Bell et al., 2012; Froehlich, 2010; Froehlich and Li, 1991; Murphy et al., 2002) and have been found to meet all of the criteria of an animal model of alcoholism (Cicero, 1979). P rats are bred in the Indiana University Alcohol Research Resource Center using an "active selection" approach where rats are tested for alcohol preference in every generation.

Fifty seven alcohol-naïve male P rats, of approximately 100 days of age, from the 77th generation of selective breeding for alcohol preference were purchased from the Indiana

University Alcohol Research Resource Center. All rats were individually housed in stainless steel hanging cages located in an isolated vivarium with controlled temperature ($21 \pm 1^\circ\text{C}$) and a reverse 12-hour light/dark cycle (lights off at 0900 hours). Standard rodent chow (Laboratory Rodent Diet #7001; Harlan Teklad, Madison, WI) and water were available ad libitum throughout the study. Fluids (water and the alcohol solution) were made available in calibrated glass Richter tubes. Water was available ad libitum and alcohol was available for 2 h/d, from 0900 hours to 1100 hours, 7 days a week. Alcohol intake (mls/kg BW) was recorded daily at the end of the 2-hour alcohol access period. Water intake (mls/kg BW) and body weight were recorded twice weekly. All subjects were acclimated to individual housing for 7 weeks prior to initiation of the study. All experimental procedures were approved by the Indiana University Institutional Animal Care and Use Committee and conducted in strict compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Alcohol Solution

A 15% (v/v) alcohol solution was prepared by diluting 95% alcohol (ethanol) with distilled and deionized water. The alcohol solution and water were presented concurrently in two separate calibrated glass drinking tubes and fluid intake was recorded to the nearest milliliter. The positions of the tubes were rotated daily to prevent development of a side preference. Alcohol intake was converted from mls alcohol/kg BW to g alcohol/kg BW.

Choice of Drug Dose

We have previously found that low doses of drugs that do not reduce alcohol intake when given alone, can effectively reduce alcohol intake when combined (Froehlich et al., 2013b, 2016; Rasmussen et al., 2015). For instance, NTX alone in a dose of 15.0 mg/kg BW and VAR alone in a dose of 1.0 mg/kg BW do not decrease ongoing alcohol drinking in P rats. However when these ineffective doses of NTX and VAR are combined into a single medication (15mg/kg BW NTX+1.0 mg /kg BW VAR) the combination decreases alcohol intake (Froehlich et al., 2016). A low dose of NTX is preferable to higher doses because the potential for side effects is reduced. A low dose of VAR (1.0 mg/kg BW) is preferable to higher doses because low doses are receptor specific and do not alter food consumption in rats (O'Connor et al., 2010). VAR, like many other ligands, interacts with receptor classes as a function of dose administered. The higher the dose, the less selective VAR becomes. In doses above 3.0 mg/kg, VAR may not be specific for the $\alpha 4\beta 2$ and/or $\alpha 7$ nicotine acetylcholine receptors (Rollema et al., 2007) and a dose of 3.0 mg/kg has been reported to decrease food intake in rats (O'Connor et al., 2010).

Drug Preparation

VAR (Pfizer INT., Groton, CT) and NTX (Pfizer INT., Groton, CT) were incorporated into star-shaped pieces of flavored gelatin and were fed to the rats. This drug delivery approach is both safe and effective for prolonged daily drug delivery (Froehlich et al., 2013a, 2013b, 2015, 2016; Rasmussen et al., 2015) and is optimal for drugs that are water soluble and orally active. NTX and VAR, alone or in combination, were dissolved in deionized and distilled water and added to a sweetened gelatin solution. The solution was comprised of berry flavored Jell-O, gelatin, dextrose, sodium saccharin and Magnasweet in distilled and deionized water. NTX and VAR, expressed as free base masses, were added to the gelatin

solution to produce the following doses: 15.0 mg NTX/3.0 ml solution/kg BW, 1.0 mg VAR/3.0 ml solution/kg BW, and 15.0 mg NTX/3.0 ml solution/kg BW + 1.0 mg VAR/3.0 ml solution/kg BW. The gelatin solution, containing drug or no drug (vehicle), was aliquoted into star-shaped molds, one per rat per day, with the volume of each aliquot determined by the body weight of the rat, as previously described (Froehlich et al., 2013a, 2013b, 2015, 2016; Rasmussen et al., 2015).

Oral Drug Delivery

The gelatin stars (approximately 1.8 g each) containing NTX and VAR, alone or in combination, were fed to the rats once each day by inserting them through a hole in the front of the cage. The rats consistently ate the gelatin star within 1 minute. Cages were checked to confirm that no pieces of gelatin were dropped. If they were, which was rare, they were refed to the rat. Gelatin stars were fed each day at 1 hour prior to onset of the daily 2-hour alcohol access period because the half-lives of NTX and VAR are relatively short. The half-life of NTX in the rat is 1.8 ± 0.21 hours (Hussain et al., 1987). The half-life of VAR in the rat is 4.0 ± 0.9 hours (Obach et al., 2005). In an earlier study, we found that consumption of a flavored gelatin star with no drug (vehicle) at 1 hour prior to a daily 2-hour alcohol access period did not alter alcohol intake (Froehlich et al., 2016). In the current study, gelatin without drug (vehicle) was fed to all rats once a day for 2 days prior to the initiation of drug treatment in order to familiarize the rats with this oral drug delivery approach. We have previously used this approach for the prolonged administration of prazosin, NTX, and VAR (Froehlich et al., 2013a, 2013b, 2015, 2016).

Experimental Design

The effect of drugs on initiation of alcohol drinking was assessed in alcohol-naïve P rats that were either pretreated with drug for 2 weeks prior to onset of alcohol access or were treated with drug concomitantly with the first opportunity to drink alcohol. Rats were matched, based on body weight (g/kg BW), and assigned to drug treatment groups in a manner that ensured that the groups did not differ in body weight (mean body weight in grams/rat: pretreated NTX = 547, VAR = 546, NTX+VAR = 546, concomitant NTX = 545, VAR = 545, NTX+VAR = 547, vehicle = 547). All rats were given 3 weeks of alcohol access for 2 h/d (0900–1100) which began on the same day for all rats. Half of the rats received daily drug treatment beginning 2 weeks prior to the onset of alcohol access. The other half of the rats received daily drug treatment beginning at the onset of alcohol access (concomitant with the first opportunity to drink alcohol). Drug treatment was delivered at 1 hour prior to daily alcohol access for 3 weeks for all rats. Food and water was available ad libitum. Drug doses were 15.0 mg/kg BW for NTX alone, 1.0 mg/kg BW for VAR alone, and 15.0 mg/kg BW for NTX + 1.0 mg/kg BW for VAR in combination. Three groups received drug concomitantly with onset of alcohol access and 3 groups received drug 2 weeks prior to onset of alcohol access, for a total of 6 groups. A 7th group (VEH) received gelatin stars without drug at one hour prior to daily alcohol access for 3 weeks and treatment began concomitantly with onset of alcohol access.

After 21 days of daily drug (or vehicle) treatment, both drug treatment and alcohol access were discontinued for 1 week. Alcohol access was then reinstated, without drug treatment,

for an additional 3 weeks in all groups, in order to assess whether alcohol intake returned to baseline levels following drug termination. Following 3 weeks of drug termination, daily alcohol access (2 h/d) was continued and drug treatment (6 groups) or vehicle treatment (1 group) was reinstated for an additional 3 weeks. The experimental timeline is depicted in Figure 1.

DATA ANALYSIS

Alcohol and water intake were analyzed using 2-way repeated-measures (RM) analyses of variance (ANOVA) (treatment X day, RM on day). When sphericity could not be assumed, as indicated by a significant Mauchly's test ($p < 0.05$), Greenhouse-Geisser was used to correct the degrees of freedom. The RM ANOVAs were followed by pairwise multiple comparisons using Dunnett's multiple comparisons against a single mean (vehicle). Significance was accepted at $p < 0.05$ and data are represented as mean $\pm$ SEM.

RESULTS

Effect of Prolonged Drug Treatment on Initiation of Alcohol Drinking and Acquisition of Alcohol Preference

The RM ANOVA (with Greenhouse-Geisser correction, $\epsilon = 0.5$) for alcohol intake in alcohol-naïve P rats that received drug treatment for 2 weeks prior to onset of alcohol access, and in alcohol-naïve P rats that received drug treatment concomitant with onset of alcohol access, revealed a significant effect of treatment $F(6, 50) = 7.1$, $p < 0.001$, day $F(9.0, 450.7) = 24.8$, $p < 0.001$, and a treatment X day interaction $F(54.1, 450.7) = 1.8$, $p < 0.01$ (Fig. 2). Further analyses using Dunnett's post-hoc comparison against a single mean (vehicle) revealed that, in rats that were pretreated with drug for 2 weeks prior to onset of alcohol access, only NTX + VAR significantly decreased alcohol intake ($p < 0.01$) (Figure 2, pretreated). In rats that received drug treatment concomitant with onset of alcohol access, NTX alone, VAR alone, and NTX + VAR each significantly decreased alcohol intake ($p < 0.05$, $p < 0.001$, and $p < 0.01$, respectively) (Figure 2 concomitant).

The RM ANOVA (with Greenhouse-Geisser correction, $\epsilon = 0.6$) on water intake in alcohol-naïve rats that received drug treatment for 2 weeks prior to onset of alcohol access, and in rats that received drug treatment concomitant with onset of alcohol access, revealed a significant effect of treatment $F(6, 2.4) = 2.4$, $p < 0.05$, a significant effect of day $F(2.9, 143.2) = 35.8$, $p < 0.001$, and a significant treatment x day interaction $F(17.2, 143.2) = 2.0$, $p < 0.05$ (Table 1). However, Dunnett's post hoc against a single mean (vehicle) revealed that no individual treatment significantly altered water intake when compared with the vehicle treated group.

Effect of Prior Drug Treatment on Alcohol Intake Following Drug Termination

After one week without drug treatment or access to alcohol, daily 2-hour alcohol access was reinstated for 21 days in the absence of drug treatment (Fig. 3). The RM ANOVA (with Greenhouse-Geisser correction, $\epsilon = 0.6$) for alcohol intake in rats that previously received drug treatment for 2 weeks prior to onset of alcohol access during drug initiation and in rats that previously received drug treatment concomitant with onset of alcohol access during

drug initiation revealed a significant effect of treatment $F(6, 50) = 2.8, p < 0.05$, day $F(11.3, 564.3) = 10.1, p < 0.001$, and a treatment X day interaction $F(67.7, 564.3) = 1.4, p < 0.05$. Further analyses using Dunnett's post-hoc comparison against a single mean (vehicle) revealed that, when drug treatment had preceded onset of alcohol access by 2 weeks during drug initiation, none of the prior drug treatments continued to reduce alcohol intake following drug termination (Figure 3, pretreated). When drug had been given concomitantly with the onset of alcohol access during drug initiation, VAR ($p < 0.05$), and NTX+VAR ($p < 0.05$) continued to reduce alcohol intake following drug termination (Figure 3, concomitant).

The RM ANOVA (with Greenhouse-Geisser correction, $\epsilon = 0.7$) on water intake in rats that previously received drug treatment for 2 weeks prior to onset of alcohol access during drug initiation and in rats that previously received drug treatment concomitant with onset of alcohol access during drug initiation, revealed no significant effect of treatment, a significant effect of day $F(3.7, 184.0) = 66.8, p < 0.001$, and no treatment X day interaction (Table 1).

Effect of Reinstatement of Prolonged Drug Treatment on Alcohol Drinking and the Expression of Alcohol Preference

The RM ANOVA (with Greenhouse-Geisser correction, $\epsilon = 0.6$) on alcohol intake in rats that previously received drug treatment for 2 weeks prior to onset of alcohol access and in rats that previously received drug treatment concomitant with onset of alcohol access revealed a significant effect of treatment $F(6, 49) = 9.5, p < 0.01$, day $F(12.4, 605.3) = 17.2, p < 0.001$, and a treatment X day interaction $F(74.1, 605.3) = 1.4, p < 0.05$ (Fig. 4). Further analyses using Dunnett's post hoc comparison against a single mean (vehicle) revealed that, when drug treatment was reinstated in subjects that had previously received drug treatment for 2 weeks prior to onset of alcohol access, only NTX+VAR reduced alcohol intake during drug reinstatement ($p < 0.05$) (Figure 4, pretreated). When drug treatment was reinstated for subjects that had previously been treated with drug concomitantly with the onset of alcohol access, only NTX+VAR and NTX alone reduced alcohol intake ($p < 0.01$, and $p < 0.01$, respectively) (Figure 4 concomitant).

The RM ANOVA (with Greenhouse-Geisser correction, $\epsilon = 0.7$) on water intake, during the reinstatement of drug treatment, in rats that received drug treatment for 2 weeks prior to onset of alcohol access during drug initiation and in rats that were treated with drug concomitantly with the onset of alcohol access during drug initiation, revealed a significant effect of treatment $F(6, 49) = 2.5, p < 0.05$, and day $F(3.4, 164.5) = 48.1, p < 0.001$, but no treatment X day interaction (Table 1). However, Dunnett's post hoc against a single mean (vehicle) revealed that no individual treatment significantly altered water intake when compared with the vehicle treated group.

DISCUSSION

The fact that alcoholism is, in part, genetically determined raises a question regarding what is inherited when one inherits a predisposition toward alcohol drinking. One thing that is inherited is a difference in the response of the opioid system to alcohol. Alcohol drinking stimulates the release of beta-endorphin which mediates the euphoric effect of alcohol (feeling of intoxication) (for review see Froehlich, 1993; Froehlich and Li, 1993; 1994;

Froehlich and Wand, 1996). The beta-endorphin response to alcohol is heritable (Froehlich et al., 2000) and the response is greater in individuals with a family history of alcoholism than it is in individuals without a family history of alcoholism (Gianoulakis et al., 1996). Both NTX and VAR reduce alcohol-induced feelings of intoxication and well-being and reduce alcohol drinking (Chick et al., 2000; Childs et al., 2012; Froehlich et al., 2003; Hendershot et al., 2016; Litten et al., 2013; McKee et al., 2009; O'Malley and Froehlich, 2003; Verplaetse, et al., 2016; Volpicelli et al., 1995). NTX is more effective in decreasing alcohol drinking in individuals with a family history of alcoholism ("family history positive" or "high risk") than in individuals without a family history of alcoholism (Krishnan-Sarin et al., 2007; Monterosso et al., 2001). The results of the current study suggest that a combination of NTX+VAR may be more effective than is either drug alone in decreasing alcohol drinking in high risk individuals.

There is a strong link between family history of alcoholism, age of onset of drinking, and amount of alcohol consumed. People with a family history of alcoholism start drinking at an earlier age and drink more than do those without a family history of alcoholism (Dawson, 2000; Hill and Yuan, 1999; Jennison and Johnson, 1998). For instance, individuals with a family history of alcohol dependence are 49% more likely to initiate alcohol drinking before age 15 (Kuperman et al., 2013). These high risk individuals are also more likely to experience difficulty in avoiding early and/or heavy alcohol drinking. We have previously reported that a combination of NTX+VAR, when administered acutely or chronically, can reduce alcohol drinking in P rats that have a well-established pattern of alcohol drinking (Froehlich et al., 2016). However, it was not known whether NTX+VAR could block the acquisition of alcohol drinking in P rats that have a genetic proclivity toward high alcohol intake. This question was addressed in the current study where NTX+VAR, alone and in combination, were administered either prior to, or concomitantly with, the first opportunity to drink alcohol in alcohol-naïve rats with a genetic predisposition toward high alcohol drinking (P line). The results indicate that NTX+VAR retards the expression of a genetic predisposition toward high alcohol drinking and suggests that this combination may be useful for curtailing alcohol drinking in high risk populations (family history positive) if it is taken prior to, or concomitantly with, the first opportunity to drink alcohol.

In the current study, the acquisition of alcohol drinking in P rats was rapid and within 7 days of initial access to alcohol for 2 h/d, P rats, treated with vehicle, were drinking in excess of 1.5 g alcohol/kg BW in 2 hours. This agrees well with our prior findings in P rats (Froehlich et al., 2013a, 2013b, 2015). We had predicted that NTX+VAR would block the acquisition of alcohol drinking in P rats if given either prior to, or concomitantly with, initial access to alcohol. The results of the current study support this prediction. NTX+VAR retarded the acquisition of alcohol drinking in rats pretreated with the combination either prior to, or concomitantly with, the onset of alcohol access. NTX+VAR not only blocked the initial acquisition of high alcohol drinking but also reduced alcohol intake, when compared to vehicle, throughout 21 days of drug treatment.

The fact that low doses of NTX or VAR, when given alone, failed to reduce alcohol drinking in P rats pretreated with the drug prior to onset of alcohol drinking (Figure 2, pretreated) agrees well with our prior findings that low doses of these drugs, when given alone, fail to

reduce ongoing alcohol drinking in P rats (Froehlich et al., 2016). The fact that NTX+VAR in combination reduced the acquisition of alcohol drinking agrees with our prior findings that low doses of NTX and VAR, when combined, reduce ongoing alcohol drinking in P rats. What was surprising in the current study was the fact that a low dose NTX alone or VAR alone, as well as in combination, blocked the acquisition of alcohol drinking when given concomitantly with the first opportunity to drink alcohol (Figure 2, concomitant). The difference between the current study and our prior studies is that the rats in the current study were alcohol-naïve. It is possible that rats that have never experienced the effects of alcohol may require a smaller dose of NTX and VAR to block the acquisition of alcohol drinking, whereas, rats with established alcohol drinking behavior are more likely to continue drinking when treated with low doses of VAR or NTX, and will only decrease their drinking when doses are increased. If this is the case, one might ask why low doses of NTX and VAR alone did not decrease the acquisition of alcohol drinking in rats treated with drug for 2 weeks prior to onset of alcohol access since they too were alcohol-naïve. One could speculate that the prolonged (2 weeks) administration of NTX alone or VAR alone, prior to onset of alcohol drinking, resulted in receptor changes that interfered with the drugs ability to decrease alcohol intake. Receptor upregulation (increase in receptor number or affinity) can occur with prolonged administration of receptor ligands such as NTX or VAR. For instance, a compensatory upregulation of opioid receptors occurs in discrete brain regions after 7–8 days of naltrexone treatment (Zukin et al., 1982). Similarly, 10 days of VAR treatment induces upregulation of nicotinic acetylcholinergic receptors ($\alpha 4\beta 2$, $\alpha 3\beta 4$ and $\alpha 7$ nACh receptors) in the brain (Marks et al., 2015). Upregulation of NTX and VAR receptors might be expected to negate the suppressive effects of NTX and VAR on initiation of alcohol drinking in rats that have received prolonged drug treatment (Melmed et al., 2015) (Figure 2, pretreated).

When daily access to alcohol was continued, but drug treatment was terminated, none of the drug treatments continued to decrease alcohol intake in rats previously pretreated with the drugs for 2 weeks prior to onset of alcohol access. However, in rats that had previously received drug treatment concomitantly with onset of alcohol access, both VAR and NTX +VAR continued to decrease alcohol intake following drug termination.

When drug treatment was reinstated after a prolonged drug free period, the combination of NTX+VAR reduced alcohol intake, as it had during the drug initiation phase, in rats previously treated with drug either prior to, or concomitantly with, the first opportunity to drink alcohol. The ability of NTX+VAR to reduce drinking after a long period without drug treatment suggests that this combination might be effective if used during times of increased risk for heavy drinking. This “targeted medication” approach has previously been suggested as an optimal strategy for implementing treatment with naltrexone and nalmefene (Kranzler et al., 1997; Mann et al., 2013; Sinclair, 2001). The rapid onset of action of NTX+VAR, which was effective on the first day of drug treatment, suggests that this combination might have a favorable compliance profile since people are more likely to adhere to treatment when beneficial effects are experienced immediately (O’Brien et al., 1992).

In summary, a combination of NTX+VAR, when administered prior to, or concomitantly with, the first opportunity to drink alcohol, blocked the acquisition of alcohol drinking

during both initial access to alcohol and during a later period of alcohol access in P rats that are selectively bred for alcohol preference and high alcohol intake. Clinical studies on the potential ability of this drug combination to curtail drinking in populations at high genetic risk for the development of alcoholism may be warranted.

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REFERENCES

- American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 5th. Virginia: American Psychiatric Publishing; 2013.
- Bell RL, Sable HJ, Colombo G, Hyttia P, Rodd ZA, Lumeng L. Animal models for medications development targeting alcohol abuse using selectively bred rat lines: neurobiological and pharmacological validity. *Pharmacol biochemBe*. 2012; 103:119–155.
- Bierut LJ, Goate AM, Breslau N, Johnson EO, Bertelsen S, Fox L, Agrawal A, Bucholz KK, Gruzza R, Hesselbrock V, Kramer J. ADH1B is associated with alcohol dependence and alcohol consumption in populations of European and African ancestry. *Mol psychiatr*. 2012; 17:445–450.
- Bouchery EE, Harwood HJ, Sacks JJ, Simon CJ, Brewer RD. Economic costs of excessive alcohol consumption in the U.S. *Am J Prev Med*. 2011; 41:516–524. [PubMed: 22011424]
- Cadoret RJ, Cain CA, Grove WM. Development of alcoholism in adoptees raised apart from alcoholic biologic relatives. *Arch Gen Psychiatr*. 1980; 37:561–563. [PubMed: 7377912]
- Chick J, Anton R, Chęcinski K, Croop R, Drummond DC, Farmer R, Labriola D, Marshall J, Moncrieff J, Morgan MY, Peters T. A multicentre, randomized, double-blind, placebo-controlled trial of naltrexone in the treatment of alcohol dependence or abuse. *Alcohol alcoholism*. 2000; 35:587–593. [PubMed: 11093966]
- Childs E, Roche DJO, King AC, de Wit H. Varenicline potentiates alcohol- induced negative subjective responses and offsets impaired eye movements. *Alcohol Clin Exp Res*. 2012; 36:906–914. [PubMed: 22339626]
- Cicero, TJ. A critique of animal analogues of alcoholism. In: Majchrowicz, E., Noble, EP., editors. *Biochemistry and Pharmacology of Ethanol*. Vol. 2. New York: Plenum Press; 1979. p. 533–560.
- Cloninger CR, Bohman M, Sigvardsson S. Inheritance of alcohol abuse: cross-fostering analysis of adopted men. *Arch Gen Psychiatr*. 1981; 38:861–868. [PubMed: 7259422]
- Cotton NS. The familial incidence of alcoholism: a review. *J Stud Alcohol*. 1979; 40:89–116. [PubMed: 376949]
- Dawson DA. The link between family history and early onset alcoholism: earlier initiation of drinking or more rapid development of dependence? *J Stud Alcohol*. 2000; 61:637–646. [PubMed: 11022800]
- Edenberg HJ, Foroud T. Genetics and alcoholism. *Nat Rev Gastroentero*. 2013; 10:487–494.
- Falk DE, Castle IJ, Ryan M, Fertig J, Litten RZ. Moderators of varenicline treatment effects in a double-blind, placebo-controlled trial for alcohol dependence: an exploratory analysis. *J Addict Med*. 2015; 9:296–303. [PubMed: 26083958]
- Fergusson DM, Boden JM, Horwood JL. Alcohol misuse and psychosocial outcomes in young adulthood: Results from a longitudinal birth cohort studied to age 30. *Drug Alcohol Depend*. 2013; 133:513–519. [PubMed: 23931962]
- Froehlich, JC. Interactions between alcohol and the endogenous opioid system, in *Alcohol and the Endocrine System*, Monograph. Zakhari, S., editor. Vol. 23. National Institute on Alcohol Abuse and Alcoholism Research Monograph Series, U.S. Department of Health and Human Services, NIH Publication; 1993. p. 21–37.

- Froehlich JC. What aspects of human alcohol use disorders can be modeled using selectively bred rat lines? *Subst Use Misuse*. 2010; 45:1727–1741. [PubMed: 20590397]
- Froehlich JC, Fischer SM, Dilley JE, Nicholson ER, Smith TN, Filosa NJ, Rademacher LC. Combining varenicline (Chantix) with naltrexone decreases alcohol drinking more effectively than does either drug alone in a rodent model of alcoholism. *Alcohol Clin Exp Res*. 2016; 40:1961–1970. [PubMed: 27469281]
- Froehlich JC, Hausauer BJ, Federoff DL, Fischer SM, Rasmussen DD. Prazosin reduces alcohol drinking throughout prolonged treatment and blocks the initiation of drinking in rats selectively bred for high alcohol intake. *Alcohol Clin Exp Res*. 2013a; 37:1552–1560. [PubMed: 23731093]
- Froehlich JC, Hausauer B, Fischer S, Wise B, Rasmussen DD. Prazosin Reduces Alcohol Intake in an Animal Model of Alcohol Relapse. *Alcohol Clin Exp Res*. 2015; 39:1538–1546. [PubMed: 26207767]
- Froehlich JC, Hausauer BJ, Rasmussen DD. Combining naltrexone and prazosin in a single oral medication decreases alcohol drinking more effectively than does either drug alone. *Alcohol Clin Exp Res*. 2013b; 37:1763–1770. [PubMed: 23875623]
- Froehlich JC, Li T-K. Animal models of the study of alcoholism: utility of selected lines. *J Addict Dis*. 1991; 10:61–71. [PubMed: 2065119]
- Froehlich, JC., Li, T-K. Opioid involvement in alcohol drinking. In: Strand, FL, Beckwith, W, Chronwall, B., Sandman, CA., editors. *Models of Neuropeptide Action*. Vol. 739. New York: Ann NY Acad Sci; 1994. p. 156-167.
- Froehlich, JC., Li, T-K. Opioid peptides, in *Recent Developments in Alcoholism*. In: Galanter, M., editor. *Ten Years of Progress*. Vol. 11. New York: Plenum Publishing Co; 1993. p. 187-205.
- Froehlich JC, O'Malley S, Hyttia P, Davidson D, Farren C. Preclinical and clinical studies on naltrexone: what have they taught each other? *Alcohol Clin Exp Res*. 2003; 27:533–539. [PubMed: 12658121]
- Froehlich JC, Wand G. The neurobiology of ethanol/opioid interactions in ethanol reinforcement. *Alcohol Clin Exp Res*. 1996; 20:181–186.
- Froehlich JC, Zink RW, Christian JC, Li T-K. Analysis of heritability of hormonal responses to alcohol in twins: Beta endorphin as a potential biomarker of genetic risk for alcoholism. *Alcohol Clin Exp Res*. 2000; 24:265–277. [PubMed: 10776662]
- Fucito LM, Toll BA, Wu R, Romano DM, Tek E, O'Malley SS. A preliminary investigation of varenicline for heavy drinking smokers. *Psychopharmacology*. 2011; 215:655–663. [PubMed: 21221531]
- Gianoulakis C, Krishnan B, Thavundayil J. Enhanced sensitivity of pituitary beta- endorphin to ethanol in subjects at high risk of alcoholism. *Arch Gen Psychiat*. 1996; 53:250–257. [PubMed: 8611062]
- Goodwin DW, Schulsinger F, Hermansen L. Alcohol problems in adoptees raised apart from alcoholic biological parents. *Arch Gen Psychiat*. 1973; 28:238–243. [PubMed: 4684290]
- Goodwin DW, Schulsinger F, Moller N. Drinking problems in adopted and nonadopted sons of alcoholics. *Arch Gen Psychiat*. 1974; 31:164–169. [PubMed: 4851437]
- Hendershot CS, Wardell JD, Samokhvalov AV, Rehm J. Effects of naltrexone on alcohol self-administration and craving: meta-analysis of human laboratory studies. *Addict Biol*. 2016
- Hill SY, Yuan H. Familial density of alcoholism and onset of adolescent drinking. *J Stud Alcohol*. 1999; 60:7–17. [PubMed: 10096304]
- Hussain MA, Aungst BJ, Kearney A, Shefter E. Buccal and oral bioavailability of naloxone and naltrexone in rats. *Int J Pharm*. 1987; 36:127–130.
- Jennison KM, Johnson KA. Alcohol dependence in adult children of alcoholics: longitudinal evidence of early risk. *J Drug Educ*. 1998; 28:19–37. [PubMed: 9567578]
- Kranzler HR, Tennen H, Penta C, Bohn MJ. Targeted naltrexone treatment of early problem drinkers. *Addict Behav*. 1997; 22:431–436. [PubMed: 9183513]
- Krishnan-Sarin S, Krystal JH, Shi J, Pittman B, O'Malley SS. Family history of alcoholism influences naltrexone-induced reduction in alcohol drinking. *Biol Psychiat*. 2007; 62:694–697. [PubMed: 17336941]
- Krystal JH, Cramer JA, Krol WF, Kirk GF, Rosenheck RA. Naltrexone in the treatment of alcohol dependence. *New Engl J Med*. 2001; 345:1734–1739. [PubMed: 11742047]

- Kuperman S, Chan G, Kramer JR, Wetherill L, Buholz KK, Dick D, Hesselbrock V, Projesz B, Rangaswamy M, Schudckit M. A model to determine the likely age of an adolescents first drink. *Pediatrics*. 2013; 131:242–248. [PubMed: 23296431]
- Li T-K, Lumeng L, McBride WJ, Waller MB, Hawkins TD. Progress toward a voluntary oral consumption model of alcoholism. *Drug Alcohol Depend*. 1979; 4:43–60.
- Litten RZ, Ryan ML, Fertig JB, Falk DE, Johnson B, Dunn KE, Green AI, Pettinati HM, Ciraulo DA, Sarid-Segal O, Kampman K, Brunette MF, Strain EC, Tiourine NA, Ransom J, Scott C, Stout R. A double-blind, placebo- controlled trial assessing the efficacy of varenicline tartrate for alcohol dependence. *J Addict Med*. 2013; 7:277–286. [PubMed: 23728065]
- Mann K, Bladström A, Torup L, Gual A, van den Brink W. Extending the treatment options in alcohol dependence: a randomized controlled study of as-needed nalmefene. *Biol Psychiat*. 2013; 73:706–713. [PubMed: 23237314]
- Marks MJ, O’Neil HC, Wyndalda-Camozzi KM, Ortix NC, Simmons EE, Short CA, Butt CM, McIntosh JM, Grady SR. Chronic treatment with varenicline changes expression of four nAChR binding sites in mice. *Neuropharmacology*. 2015; 99:142–155. [PubMed: 26192545]
- McKee SA, Harrison ELR, O’Malley SS, Krishnan-Sarin S, Shi J, Tetrault JM, Picciotto MR, Petrakis IL, Estevez N, Balchunas E. Varenicline reduces alcohol self-administration in heavy-drinking smokers. *Biol Psychiat*. 2009; 66:185–190. [PubMed: 19249750]
- Melmed, S., Polonsky, KS., Larsen, PR., Kronenberg, HM. *Williams Textbook of Endocrinology*. 13th. Amsterdam: Elsevier; 2015.
- Mitchell JM, Teague CH, Kayser AS, Bartlett SE, Fields HL. Varenicline decreases alcohol consumption in heavy-drinking smokers. *Psychopharmacology*. 2012; 223:299–306. [PubMed: 22547331]
- Mitchell JM, Teague CH, Kayser AS, Bartlett SE, Fields HL. Varenicline decreases alcohol consumption in heavy-drinking smokers. *Psychopharmacology*. 2012; 223:299–306. [PubMed: 22547331]
- Monterosso JR, Flannery BA, Pettinati HM, Oslin DW, Rukstalis M, O’Brien CP, Volpicelli JR. Predicting treatment response to naltrexone: the influence of craving and family history. *Am J Addiction*. 2001; 10:258–268.
- Murphy JM, Stewart RB, Bell RL, Badia-Elder NE, Carr LG, McBride WJ, Lumeng L, Li T-K. Phenotypic and genotypic characterization of the Indiana University rat lines selectively bred for high and low alcohol preference. *Behav Genet*. 2002; 32:363–388. [PubMed: 12405517]
- Nurnberger JJ Jr, Wiegand R, Bucholz K, O’Connor S, Meyer ET, Reich T, Rice J, Schuckit M, King L, Petti T, Bierut L, Hinrichs AL, Kuperman S, Hesselbrock V, Projesz B. A family study of alcohol dependence: coaggregation of multiple disorders in relatives of alcohol-dependent probands. *Arch Gen Psychiatry*. 2004 Dec;61:1246–1256. Erratum in: *Arch Gen Psychiatry* Aug; 62:848. [PubMed: 15583116]
- Obach RS, Reed-Hagen AE, Krueger SS, Obach BJ, O’Connell TN, Zandi KS, Miller S, Coe JW. Metabolism and disposition of varenicline, a selective $\alpha 4\beta 2$ acetylcholine receptor partial agonist, in vivo and in vitro. *Drug Metab Dispos*. 2005; 34:121–130. [PubMed: 16221753]
- O’Brien MK, Petrie K, Raeburn J. Adherence to medication regimens: updating a complex medical issue. *Med Care Res Rev*. 1992; 49:435–454.
- O’Connor EC, Parker D, Rollema H, Mead AN. The $\alpha 4\beta 2$ nicotinic acetylcholine-receptor partial agonist varenicline inhibits both nicotine self-administration following repeated dosing and reinstatement of nicotine seeking in rats. *Psychopharmacology*. 2010; 208:365–376. [PubMed: 19967529]
- O’Malley SS, Adam JJ, Rode S, Rounsaville BJ. Experience of a “slip” among alcoholics treated with naltrexone or placebo. *Amer J Psychiat*. 1996; 153:281–283. [PubMed: 8561215]
- O’Malley SS, Corbin WR, Leeman RF, DeMartini KS, Fucito LM, Ikomi J, Romano DM, Wu R, Toll BA, Sher KJ. Reduction of alcohol drinking in young adults by naltrexone: a double-blind, placebo-controlled, randomized clinical trial of efficacy and safety. *The J clin psychiat*. 2015; 76:207–213.

- O'Malley, S., Froehlich, JC. Advances in the use of naltrexone: an integration of preclinical and clinical findings. In: Galanter, M., editor. *Recent Developments in Alcoholism XVI: Research on Alcoholism Treatment*. New York, NY: Kluwer Academic/Plenum Publishers; 2003. p. 217-245.
- Rasmussen DD, Kincaid CL, Froehlich JC. Prazosin + naltrexone decreases alcohol drinking more effectively than does either drug alone in P rats with a protracted history of extensive voluntary alcohol drinking, dependence and multiple withdrawals. *Alcohol Clin Exp Res*. 2015; 39:1832–1841. [PubMed: 26260061]
- Ray LA, Courtney KE, Ghahremani DG, Miotto K, Brody A, London ED. Varenicline, low dose naltrexone, and their combination for heavy-drinking smokers: human laboratory findings. *Psychopharmacology*. 2014; 231:3843–3853. [PubMed: 24733235]
- Ray LA, Courtney KE, Ghahremani DG, Miotto K, Brody A, London ED. Varenicline, naltrexone, and their combination for heavy-drinking smokers: preliminary neuroimaging findings. *Am J Drug Alcohol Abuse*. 2015; 41:35–44. [PubMed: 24949564]
- Roche DJ, Bujarski S, Hartwell E, Green R, Ray LA. Combined varenicline and naltrexone treatment reduces smoking topography intensity in heavy-drinking smokers. *Pharmacol Biochem Behav*. 2015; 134:92–98. [PubMed: 25933795]
- Rollema H, Chambers L, Coe J, Glowa J, Hurst R, Lebel L, Mansbach RS, Mather RJ, Rovetti CC, Sands SB, Schaeffer E, Schulz DW, Tingley FD3rd, Williams KE. Pharmacological profile of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. *Neuropharmacology*. 2007; 52:985–994. [PubMed: 17157884]
- Schacht JP, Anton RF, Randall PK, Li X, Henderson S, Myrick H. Varenicline effects on drinking, craving and neural reward processing among non-treatment-seeking alcohol- dependent individuals. *Psychopharmacology*. 2014; 231:3799–3807. [PubMed: 24647921]
- Sinclair JD. Evidence about the use of naltrexone and for different ways of using it in the treatment of alcoholism. *Alcohol Alcoholism*. 2001; 36:2–10. [PubMed: 11139409]
- Steensland P, Simms JA, Holgate J, Richards JK, Bartlett SE. Varenicline, an $\alpha 4\beta 2$ nicotinic acetylcholine receptor partial agonist, selectively decreases ethanol consumption and seeking. *Proc Natl Acad Sci USA*. 2007; 104:12518–12523. [PubMed: 17626178]
- Verplaetse TL, Pittman BP, Shi JM, Tetraault JM, Coppola S, McKee SA. Effect of Varenicline Combined with High-Dose Alcohol on Craving, Subjective Intoxication, Perceptual Motor Response, and Executive Cognitive Function in Adults with Alcohol Use Disorders: Preliminary Findings. *Alcohol Clin Exp Res*. 2016; 40:1567–1576. [PubMed: 27246567]
- Volpicelli JR, Alterman AI, Hayashida M, O'Brien CP. Naltrexone and the treatment of alcohol dependence. *Arch Gen Psychiat*. 1992; 49:876–880. [PubMed: 1345133]
- Volpicelli JR, Watson NT, King AC, Sherman CE, O'Brien CP. Effect of naltrexone on alcohol "high" in alcoholics. *Am J Psychiat*. 1995; 152:613–615. [PubMed: 7694913]
- White A, Hingson R. The burden of alcohol use. *Alcohol Res*. 2014; 35:201–218.
- Zukin RS, Sugarman JR, Fitz-Syage ML, Gardner EL, Zukin SR, Gintzler AR. Naltrexone- induced opiate receptor supersensitivity. *Brain Res*. 1982; 245:285–292. [PubMed: 6289965]

EXPERIMENTAL TIMELINE

DRUG INITIATION (Drug + Alcohol Access)		DRUG TERMINATION (Alcohol Access, No Drug Treatment)	DRUG REINSTATEMENT (Drug + Alcohol Access)
0.0 mg VEH 15.0 mg NTX - Pretreated 1.0 mg VAR - Pretreated 15.0 mg NTX + 1.0 mg VAR - Pretreated 15.0 mg NTX - Concomitant 1.0 mg VAR - Concomitant 15.0 mg NTX + 1.0 mg VAR - Concomitant	No Alcohol Access No Drug Treatment		0.0 mg VEH 15.0 mg NTX - Pretreated 1.0 mg VAR - Pretreated 15.0 mg NTX + 1.0 mg VAR - Pretreated 15.0 mg NTX - Concomitant 1.0 mg VAR - Concomitant 15.0 mg NTX + 1.0 mg VAR - Concomitant
3 WEEKS	1 WEEK	3 WEEKS	3 WEEKS

Figure 1.
Experimental Timeline

Figure 2.

Effect of naltrexone (NTX), varenicline (VAR), or NTX+VAR, on the acquisition of alcohol drinking in alcohol-naïve P rats. Drugs were given either 2 weeks prior to (pretreated), or concomitantly with (concomitant), the onset of 3 weeks of alcohol access. The vehicle group is depicted with a solid line in the concomitant panel and is also transposed as a grey line in the pretreated panel to facilitate visual comparison of the vehicle treated group and the 6 drug treated groups. Each point represents the mean $\pm$ SEM.

Figure 3.

Alcohol intake during the 3 weeks following termination of naltrexone (NTX), varenicline (VAR), or NTX+VAR treatment in P rats that had been given drug either 2 weeks prior to (pretreated), or concomitantly with (concomitant), the onset of alcohol access during drug initiation. The vehicle group is depicted with a solid line in the concomitant panel and is also transposed as a grey line in the pretreated panel to facilitate visual comparison of the vehicle treated group and the 6 drug treated groups. Each point represents the mean $\pm$ SEM.

Figure 4. Effect of naltrexone (NTX), varenicline (VAR), or NTX+VAR on alcohol intake during 3 weeks of drug reinstatement in rats that had been given drug for either 2 weeks prior to (pretreated), or concomitantly with (concomitant), the onset of alcohol access during drug initiation. The vehicle group is depicted with a solid line in the concomitant panel and is also transposed as a grey line in the pretreated panel to facilitate visual comparison of the vehicle treated group and the 6 drug treated groups. Each point represents the mean $\pm$ SEM.

Table 1
Effect of Drug Treatment on Water Intake (mls/kg BW)

The effect of naltrexone (NTX) (15.0 mg/kg BW), varenicline (VAR) (1.0 mg/kg BW), or NTX (15.0 mg/kg BW)+VAR (1.0 mg/kg BW) on water intake. Water consumption was recorded twice weekly, and values represent the average water intake (ml/kg BW $\pm$ SEM) in each treatment group during drug initiation, drug termination, and drug reinstatement.

Treatment	Drug Initiation	Drug Termination	Drug Reinstatement
Vehicle	46.3 $\pm$ 2.1	39.6 $\pm$ 1.9	36.0 $\pm$ 1.3
NTX Pretreated	47.6 $\pm$ 2.0	41.3 $\pm$ 2.1	38.0 $\pm$ 1.5
VAR Pretreated	46.3 $\pm$ 1.9	42.4 $\pm$ 2.0	36.9 $\pm$ 1.4
NTX+VAR Pretreated	55.3 $\pm$ 0.9	48.4 $\pm$ 1.5	43.6 $\pm$ 1.6
NTX Concomitant	50.0 $\pm$ 1.9	44.4 $\pm$ 1.8	39.0 $\pm$ 1.5
VAR Concomitant	56.2 $\pm$ 1.0	47.7 $\pm$ 1.8	42.9 $\pm$ 1.6
NTX+VAR Concomitant	49.7 $\pm$ 1.2	45.4 $\pm$ 1.5	41.4 $\pm$ 1.5