Cognitive-enhancing effects of acetylcholine receptor agonists in group-housed cynomolgus monkeys who drink ethanol

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Abstract

The cognitive impairments that are often observed in patients with alcohol use disorder (AUD) partially contribute to the extremely low rates of treatment initiation and adherence. Brain acetylcholine receptors (AChR) mediate and modulate cognitive and reward-related behavior and their distribution can be altered by long-term heavy drinking. Therefore, AChRs are promising pharmacotherapeutic targets for treating the cognitive symptoms of AUD. In the present study, the pro-cognitive efficacy of two AChR agonists, xanomeline and varenicline, were evaluated in group-housed monkeys who self-administered ethanol for more than one year. The muscarinic AChR antagonist scopolamine was used to disrupt performance of a serial stimulus discrimination and reversal (SDR) task designed to probe cognitive flexibility, defined as the ability to modify a previously learned behavior in response to a change in reinforcement contingencies. The ability of xanomeline and varenicline to remediate the disruptive effects of scopolamine was compared between dominant and subordinate monkeys, with lighter and heavier drinking histories, respectively. We hypothesized that subordinate monkeys would be more sensitive to all three drugs. Scopolamine dose-dependently impaired performance on the serial SDR task in all monkeys at doses lower than those that produced non-specific impairments (e.g., sedation); its potency did not differ between dominant and subordinate monkeys. However, both AChR agonists were effective in remediating the scopolamine-induced deficit in subordinate monkeys, but not in dominant monkeys. These findings suggest xanomeline and varenicline may be effective for enhancing cognitive flexibility in individuals with a history of heavy drinking.

Significance statement: Pro-cognitive effects of two acetylcholine (Ach) receptor agonists were assessed in group-housed monkeys who had several years’ experience drinking ethanol. The muscarinic ACh receptor agonist xanomeline and the nicotinic ACh receptor agonist varenicline reversed a cognitive deficit induced by the muscarinic ACh receptor antagonist scopolamine.
However, this effect was observed only in lower-ranking (subordinate) monkeys and not higher-ranking (dominant monkeys). Results suggest that ACh agonists may effectively remediate alcohol-induced cognitive deficits in a subpopulation of those with alcohol use disorder.
Introduction

Alcohol use disorder (AUD) is associated with cognitive impairments (Le Berre et al., 2017; Sullivan et al., 2010) attributed to alcohol-induced changes in frontocortical brain regions (e.g., Berre et al., 2014; Fan et al., 2023; Pfefferbaum et al., 1997). Of particular concern are impairments in cognitive flexibility—the ability to modify a previously learned behavior in response to changes in reinforcement contingencies (Dajani & Uddin, 2015). Individuals with long-term drinking histories show decreased performance on tasks that probe cognitive flexibility, such as stimulus discrimination and reversal (SDR), intradimensional/extradimensional (ID/ED) set shifting, Stroop and Wisconsin Card Sorting tasks (e.g., Fein et al., 2006; Sullivan et al., 1993). Impairments in cognitive flexibility can reduce motivation to seek AUD treatment, contributing to low treatment rates (~4.6% in 2020; Bates et al., 2002; Le Berre et al., 2012; SAMHSA, 2021). Moreover, length of abstinence is positively correlated with remediation of alcohol-induced cognitive impairments (Andó et al., 2012; Fein et al., 2006; Le Berre et al., 2017). These findings highlight the potential of cognitive enhancement as a treatment approach (Bates et al., 2002; Sofuoglu et al., 2013).

Brain acetylcholine (ACh) systems mediate complex cognitive processes. ACh receptors (AChRs) are distributed widely and cholinergic neurons innervate frontocortical areas that mediate cognitive flexibility (Everitt & Robbins, 1997; Prado et al., 2017). For these reasons, the ACh system has become a target for pharmacotherapies to remediate cognitive deficits in substance use disorders (Chatterjee & Bartlett, 2010; Walker et al., 2023). Repurposing existing pharmacotherapies that have been FDA-approved for other indications may be an advantageous approach, as their known pharmacokinetics, pharmacodynamics and safety profiles may accelerate approval. Xanomeline, a M1/M4 muscarinic AChR (mAChR)-preferring agonist, and varenicline, an α4β2/α7 nicotinic AChR (nAChR) agonist, are ideal candidates for repurposing, as previous research has indicated they have pro-cognitive effects. Xanomeline...
showed promise in managing dementia related to Alzheimer’s disease, but peripheral side effects prevented FDA approval (Brannan et al., 2020). Xanomeline plus trospium chloride (a peripherally restricted mAChR antagonist) is nearing FDA approval as a combination medication for treating cognitive symptoms of schizophrenia. Varenicline, an FDA-approved medication for smoking cessation, dose-dependently improved measures of working memory in AUD patients; other pro-cognitive effects have been reported in humans and nonhuman primates (NHPs) with histories of methamphetamine or cocaine self-administration, respectively (Gould et al., 2013; Kalechstein et al., 2014; Roberts & McKee, 2018). To date, however, no studies have investigated the therapeutic effects of xanomeline or varenicline for improving cognitive flexibility in individuals with long-term alcohol drinking histories.

Evaluating putative pharmacotherapies in humans can be confounded by variables such as polysubstance use, comorbid psychiatric disorders and past and current environmental experiences and conditions that can be controlled in laboratory animal models. NHPs are the most translationally relevant laboratory animal regarding brain structure and function, pharmacokinetics, metabolism, genetics, etc. (Phillips et al., 2014; Weerts et al., 2007). Importantly, NHPs will drink ethanol in clinically relevant patterns (e.g., Baker et al., 2014). Furthermore, social and environmental factors that influence alcohol-drinking trajectories can be incorporated into NHP models. Group-housed NHPs form social dominance hierarchies and experience a continuum from social enrichment in the highest-ranked (dominant) monkeys to chronic social stress in the lowest-ranked (subordinate) monkeys (Czoty et al., 2009; Nader et al., 2012). Like humans who experience chronic social stress, subordinate monkeys have increased rates of morbidity and mortality and consume more ethanol than dominant monkeys (Galbo et al., 2022; Helms et al., 2012; McKenzie-Quirk & Miczek, 2008; Sapolsky, 2005). For these reasons, group-housed monkeys with varied ethanol drinking histories more accurately reflect variation in human subjects.
Cognitive flexibility, which involves changing one’s behavior to achieve new goals and as such is critical to AUD treatment (Bates et al., 2002; Le Berre et al., 2017), can be probed in NHPs with an SDR task that has been reverse-translated from those validated in humans (Jentsch & Taylor, 1999; Weed et al., 1999; Kangas, 2022). Scopolamine, a non-selective mAChR antagonist, has been used to pharmacologically induce a cognitive deficit; the ability of drugs to reverse a scopolamine-induced deficit can be used as a measure of pro-cognitive efficacy (Klinkenberg & Blokland, 2010; Buccafusco, 2009; Buccafusco et al., 2008). In the present studies, a scopolamine reversal paradigm was used to evaluate pro-cognitive effects of xanomeline and varenicline in group-housed monkeys with ethanol self-administration histories. We hypothesized that, compared to dominant-ranked monkeys, heavy drinking subordinate monkeys would be more sensitive to the cognitive-disrupting effects of scopolamine and to the ability of xanomeline and varenicline to reverse a scopolamine-induced deficit.
Materials and Methods

Subjects. Twelve adult male cynomolgus monkeys (*Macaca fascicularis*, 12.5 ± 2.1 years old at the start of the study) served as subjects. Monkeys lived in groups of four or pairs in stainless-steel cages (Allentown Caging; Allentown, NJ) in which water was available ad libitum. A 14:10 light-dark cycle was in place with lights on at 6:00 a.m. and off and 8:00 p.m. Monkeys were weighed every other week and fed enough food (Grain-Based Dustless Precision Pellets Bio-Serve; Flemington, NJ), fresh fruit and vegetables daily to maintain healthy body weights without becoming obese, as determined by daily inspection and periodic veterinary examinations. Body weights did not change significantly during these studies. Animal housing, handling and all experimental procedures were performed in accordance with the 2011 edition of the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Wake Forest University. Environmental enrichment was provided as outlined in the Committee's Nonhuman Primate Environmental Enrichment Plan.

Social rank determination. Details of the determination of social rank in this cohort (dominant, n=6, or subordinate, n=6) were previously described (Galbo et al., 2022). Briefly, monkeys were randomly assigned by weight to three pens (Pens A, B and C). Social ranks were determined over 12 weeks of video observation of dyadic aggressive and submissive interactions (Galbo et al., 2022; Morgan et al., 2000). In each Pen, the two highest-ranked monkeys (#1 and #2) were considered dominant, whereas the two lowest ranked (#3 and #4) were considered subordinate. For the duration of the study, social rank was assessed by observing interactions between all monkeys in the pen when group housed. While social ranks remained stable in Pens A and C, a fight occurred in Pen B approximately 19 months following their initial introduction and 11 weeks into the ethanol self-administration period. Attempts at reintroduction were ultimately deemed unsuccessful due to continued aggression. For the safety of the monkeys, they were re-grouped.
into pairs and the dominant and subordinate monkeys in each pair were identified (dominant: 8501 and 8528; subordinate: 8493 and 8527). Thus, at the outset of the present studies, ranks had been stable in Pens A and C for at least 1.5 years, and in the Pen B pairs for at least 1 year. For the duration of the present studies, drug administration did not cause any observable changes in aggressive, submissive or affiliate behaviors and social ranks remained stable.

**Ethanol self-administration.** Monkeys were separated into quadrants of the home-cage using mesh partitions and trained to self-administer ethanol via an operant drinking panel permanently affixed to the side of the quadrant using methods of schedule-induced polydipsia previously described (Galbo et al., 2022; Vivian et al., 2001). Subsequently, they were given “open access” to self-administer ethanol or water 22 hours per day, 4-5 days per week, for one year; these ethanol self-administration data have been reported (Galbo-Thomma et al., 2023).

**Cognitive testing apparatus.** Cognitive testing was conducted using a custom-built operant panel housing a touch-sensitive monitor that temporarily mounted to the front of the home cage. A pellet dispenser (ENV-203-190IR; Med Associates Inc., St. Albans, VT) was mounted behind the monitor and powered by an Arduino Nano Microcontroller (V3.0, UCEC). Following correct responses, sucrose pellets (190 mg, Fruit Crunchies™; Bio-Serv, Flemington, NJ) were dispensed and delivered via tubing (TPI Partners, Inc., Georgetown, DE) to a modified stainless-steel box feeder (Allentown Caging, Allentown, NJ) containing a food well directly below the monitor. A custom-designed SDR task (E-Prime 3.0, Pittsburgh, PA; Microsoft Visual Studio Community 2019 V16.7.7 and Microsoft .NET Framework V4.8.04084, Redmond, WA) was displayed on a laptop (EliteBook 840 G6; Hewlett Packard, Palo Alto, CA) and duplicated to a 38.1-cm flat IP65 P-CAP display monitor (active display area 1024x768; R15L100-SPC3; TSI Touch, Uniontown, PA) built within an aluminum extrusion frame. The door of the home cage
was lifted and the frame was mounted on the cage with metal brackets, allowing the monkey direct access to the monitor without obstruction by the cage door.

**Touchscreen training.** Touchscreen training occurred early in the open-access period. Monkeys were trained to respond on the touchscreen monitor using a stimulus-fade task (E-Prime 3.0). For each trial, a one-response fixed ratio schedule of reinforcement on a training stimulus (green square with a centered fixation point) resulted in an 880-ms flash (yellow screen) and delivery of a food pellet, followed by a 5-sec inter-trial interval (ITI; black screen), whereas responding outside the boundaries of the stimulus (incorrect response) resulted in a 10-sec timeout (black screen), followed by the ITI. The training stimulus decreased in size every 20 trials, beginning with occupation of the entire screen and subsequently 75%, 50% and 25% of the screen. Monkeys acquired the task after making at least 90% correct responses on the smallest stimulus for three consecutive sessions. Five monkeys (8528, 8527, 8495, 8108 and 8500) performed modified versions of this task for a small fraction of training sessions due to slow task acquisition. The modified tasks either increased the response requirement to a two-response fixed ratio schedule of reinforcement or eliminated the two largest stimuli (100% and 75%) and increased the number of trials to 40 for two smallest stimuli (50% and 25%). An unpaired t-test was performed to determine rank-related differences in the number of sessions required meet acquisition criterion.

**Serial discrimination and reversal (SDR) task.** Monkeys performed a serial SDR task programmed with Microsoft Visual Studio Community. A gray screen with a centered fixation point was presented at the outset of each session and a single response anywhere on the screen started the session. In each session, a set of five distinctly shaped and colored stimuli were presented, one in each corner and one in the center of the screen. Stimulus shapes were randomized from 7 unique possibilities and stimulus color was randomized from 132 pre-defined
Microsoft Windows colors, ensuring a difference in hue value $\geq 60^\circ$ between all colors. One stimulus was randomly designated as the $S^+$ and a one response (touch) on this stimulus resulted in an 880-ms flash (yellow screen), the delivery of a food pellet and a 5-sec ITI. Responding on any of the other four stimuli, termed “$S^-$s, did not result in pellet delivery and instead initiated a 10-sec timeout (black screen) followed by the ITI. A trial was omitted if no response was made in 30-sec, which was followed by the timeout and ITI. The stimulus set remained the same throughout the session but the location of the stimuli on the screen shuffled randomly each trial. After making 18 correct responses within 20 consecutive trials, the response contingency “reversed” such that the $S^+$ became a $S^-$ and one of the previous $S^-$s became the $S^+$; the $S^+$ could not be repeated between two consecutive reversal blocks. All conditions and the response criterion remained the same, and when the criterion was met again, another reversal block began. Each session lasted 90-min. The primary dependent variable was the number of completed reversals in a session. At the outset of these studies, monkeys had self-administered ethanol for approximately 15 months under open-access conditions. Prior to testing scopolamine, xanomeline and varenicline, monkeys performed a single session of the SDR task and an unpaired t-test was performed to determine whether the ranks differed in performance.

**Administration of scopolamine and drug combinations.** All drug administration and cognitive testing occurred between 8:00a.m. and 3:00p.m. Once xanomeline/varenicline experiments started, the drinking regimen was changed such that two days of drinking under open access conditions (22 hrs/day) alternated with two non-drinking days. Cognitive testing occurred on the second non-drinking day (i.e., the fourth day of a four-day cycle). The ability of xanomeline and varenicline to reverse a scopolamine-induced cognitive deficit (a decrease in the number of reversals a monkey completed within a SDR session) was determined. In Experiment 1, saline
or sterile water was administered at least twice to determine baseline performance, and then intermittently throughout the remainder of the experiment. Dose-effect curves for scopolamine bromide (0.003-0.56 mg/kg; 30-min pre-treatment; SCOP; Sigma Aldrich, St. Louis, Missouri, United States) and xanomeline hydrochloride (0.01-0.3 mg/kg; 30-min pretreatment; Research Triangle Institute, Research Triangle Park, NC) were determined using a mixed-order design. Subsequently, the highest doses of xanomeline that did not produce severe cholinergic side effects (see Cholinergic side effects) were co-administered with a dose(s) of scopolamine that produced a ≥50% decrease in performance from the vehicle baseline (termed the “best dose” of scopolamine). Dose combinations were administered in mixed order. It took approximately 3.5 months to complete Experiment 1. Immediately thereafter, Experiment 2 was conducted using the same 4-day cycle (2 days of open-access ethanol drinking one day off, testing on the fourth day) except that varenicline dihydrochloride (0.003-0.3 mg/kg; Research Triangle Institute, 75-min pretreatment) was tested rather than xanomeline. It took approximately 5.5 months to complete Experiment 2.

Data analysis: Assessment of effects of practice and each drug alone. To determine whether dominant or subordinate monkeys' performance on the task improved over time, the number of reversals made following vehicle administration in Experiments 1 versus 2 were compared using a 2-way repeated measures ANOVA with rank (dominant, subordinate) and experiment (1, 2) as factors, followed by a post-hoc Sidak multiple comparisons analysis. The same analysis was performed to assess rank- or time-related differences in sensitivity to scopolamine by comparing scopolamine ED$_{50}$ values, defined as the interpolated scopolamine dose that produced a 50% decrease in performance accuracy. To determine whether scopolamine decreased the number of reversals by increasing errors (indicative of a decrease in cognitive function) or omissions (which could be caused by non-specific effects such as sedation), a “best dose” of scopolamine was selected as the highest dose that produced a ≥50% reduction in performance accuracy.
without producing a substantial increase in omissions or the presence of cholinergic side effects. A 3-way repeated measures ANOVA was then performed on the mean percent of trials on which errors occurred and mean percent of trials on which omissions occurred for each monkey’s vehicle baseline and “best dose” of scopolamine in Experiments 1 and 2. Specifically, in the ANOVA, rank (dominant, subordinate), treatment (vehicle, scopolamine) and time (Experiment 1 or 2) were factors. The ANOVA was followed by a post-hoc Sidak multiple comparisons tests. The effects of xanomeline or varenicline administered alone were assessed with separate 2-way ANOVAs with rank and drug dose as factors.

Data analysis: Assessment of remediation of the scopolamine-induced deficit. The primary measure used to assess potential rank-related differences in the efficacy of xanomeline and varenicline in remediating a scopolamine-induced deficit was the amount of the scopolamine deficit that was remediayed after administration of the most effective combination, expressed as a percent of the scopolamine deficit. For example, if the baseline was 10 reversals and scopolamine decreased reversals to 2 (an 80% decrease) and after the most effective combination (“best combination”), there were 6 reversals (a 40% decrease from baseline), percent remediation was calculated to be 50% because the combination remediayed half of the deficit produced by that dose of scopolamine. Mean percent remediation for dominant vs. subordinate monkeys was compared using an unpaired t-test for each drug. We also determined the mean percent of errors and omissions of total trials for all best combinations. Unpaired t-tests were performed to determine whether there were any rank-related differences in these measures.

Cholinergic side effects. To detect peripheral cholinergic side effects, monkeys were observed at the start and end of each SDR session. Using a list of potential effects adapted from Vardigan et al. (2015), the absence or presence of emesis, salivation and changes in normal activity were
recorded (Table 1). If a monkey displayed recumbency, veterinary staff was contacted and anti-nausea medication was administered. Typically, when recumbency was observed, very few trials, if any, were completed; these sessions were excluded from analyses.

Results

Touchscreen training and initial SDR performance. Monkeys had similar lifetime ethanol intakes when touchscreen training began and once all monkeys met acquisition criteria of the stimulus-fade task (mean ± SD: 105.0 ± 14.1 g/kg). Subordinate monkeys took a significantly higher number of sessions to meet the touchscreen training criterion compared to dominant monkeys (t_{10}=2.68, p<0.05; Fig. 1). After one year of open access ethanol self-administration, despite a significant difference in ethanol intake (reported in Galbo-Thomma et al., 2023 and Figure 2), dominant and subordinate monkeys did not differ in SDR performance (t\textsubscript{(10)}=1.38, p=0.20; Fig. 2)

Assessment of effects of practice and each drug alone. To assess whether performance of the task increased due to practice over the course of the present studies, the number of reversals completed following vehicle administration was compared between Experiments 1 and 2 (Fig. 3). A 2-way repeated measures ANOVA revealed a statistically significant main effect of time (F\textsubscript{(1,10)}=42.44, p<0.0001), but not of rank (F\textsubscript{(1,10)}=0.03, p=0.88), and no interaction (F\textsubscript{(1,10)}=3.32, p=0.10). A post-hoc Sidak multiple comparisons test indicated a significant difference in number of reversals completed for both dominant (p<0.05) and subordinate monkeys (p<0.0001). At neither time point was the number of reversals after vehicle administration significantly correlated with lifetime ethanol intake (Experiment 1: Pearson r=-0.209, p=0.515; Experiment 2: Pearson r=0.108, p=0.297). Although cognitive performance increased over time, the potency of scopolamine in inducing a cognitive deficit did not change. There was no significant rank-related difference in this effect of scopolamine (F\textsubscript{(1,10)}=0.03, p=0.86) and no change over time in scopolamine ED\textsubscript{50} in either group (p=0.74 in dominants and p=0.98 in subordinates; Fig. 4). At
neither time point was the potency of scopolamine significantly correlated with lifetime ethanol intake (Experiment 1: Pearson $r=-0.104$, $p=0.308$; Experiment 2: Pearson $r=0.015$, $p=0.704$).

Next, whether scopolamine reduced performance due to a decrease in cognitive flexibility (increased percent of trials as errors) or by non-specific behavioral disruption (increased numbers of omissions) was examined (Fig. 5). For percent errors, there was a main effect of time ($F_{(1,10)}=5.67$, $p<0.05$) and rank ($F_{(1,10)}=32.70$, $p<0.001$), but not vehicle/scopolamine treatment ($F_{(1,10)}=2.46$, $p=0.15$), and a significant interaction between time, treatment and rank ($F_{(1,10)}=6.44$, $p<0.05$). Post-hoc analysis revealed a significant difference for subordinates in percent errors between vehicle and scopolamine in Experiment 2 ($p=0.001$). For percent omissions, there was only a significant main effect of rank ($F_{(1,10)}=14.26$, $p<0.005$; time: $F_{(1,10)}=1.36$, $p=0.8827$, treatment: $F_{(1,10)}=0.67$, $p=0.43$), and the post-hoc analysis did not reveal any significant differences. The effects of xanomeline and varenicline when administered alone are depicted in Figure 6. Although individual subject variability was observed, the average effects of these drugs on reversals completed did not differ according to social rank; the only statistically significant effect was a main effect of XML dose (XML: dose: $F_{(1.462,14.62)}=7.09$, $p<0.05$, rank: $F_{(1,10)}=0.53$, $p=0.48$, interaction: $F_{(2,20)}=0.15$, $p=0.86$; VAR: dose: $F_{(4,40)}=1.49$, $p=0.22$, rank: $F_{(1,10)}=0.26$, $p=0.62$, interaction: $F_{(4,40)}=0.27$, $p=0.90$).

Remediation of the scopolamine-induced deficit. The effects of selected doses of xanomeline alone and in combination with scopolamine are shown in supplementary figures Supp. 1 and Supp. 2 and in Table 2. For Experiment 1, an unpaired t-test revealed no social rank-related difference in percent change from the vehicle baseline produced by the “best dose” of scopolamine ($t_{(10)}=0.78$, $p=0.45$; Table 2). This observation confirms that the scopolamine doses selected for testing drug combinations were behaviorally equivalent between dominant and subordinate monkeys. In subordinate monkeys, xanomeline remediated 40-70% of the deficit produced by scopolamine in all monkeys (Table 2, Fig. Supp. 1). In contrast, a range of effects
was observed in dominant monkeys. In three dominant monkeys, xanomeline remediated 19.1%, 75.0% and 80.0% of the scopolamine induced deficit. Xanomeline had no effect in one monkey and increased the deficit in two monkeys by 14.3% and 23.2% (Table 2, Fig. Supp. 2). Because of the high degree of variability in the effects of xanomeline in dominant monkeys, the difference in effects between dominant and subordinate monkeys was not statistically significant ($t_{10}=1.75, p=0.11$; Fig. 7). When the best combination of xanomeline and scopolamine was tested, subordinate monkeys made significantly more errors than dominant monkeys (Table 2; $t_{10}=3.78, p<0.005$). The difference in omissions made between dominants (18.1 ± 8.2%) and subordinates (1.6 ± 0.9%) approached statistical significance (Table 2; $t_{10}=2.20, p=0.052$).

The effects of selected doses of varenicline alone and in combination with scopolamine are shown in supplementary Figures Supp. 3 and Supp. 4 and Table 2. As in Experiment 1, the degree to which the selected scopolamine doses produced a cognitive deficit did not differ between dominant and subordinate monkeys ($t_{10}=1.77, p=0.11$; Table 2). As seen with xanomeline, varenicline increased the number of reversals in all subordinate monkeys with a mean remediation of 61.4 ± 10.9%; however, the largest remediation observed in any dominant monkey was 20.6% (Table 2, Figs. Supp. 3 and Supp. 4). When the ranks were compared directly, the ability of varenicline to remediate the scopolamine-induced deficit was significantly greater in subordinate monkeys than in dominants ($t_{10}=4.80, p<0.001$; Fig. 7). There were no rank-related differences in errors during varenicline testing ($t_{10}=0.20, p=0.85$), but dominants made significantly more omissions compared to subordinates (Table 2; $t_{10}=2.392, p<0.05$).

**Cholinergic side effects.** Cholinergic side effects were rarely observed after administration of any drug or drug combination. Table 3 depicts the specific dose(s) and number of monkeys who experienced side effect scores of higher severity (scores for emesis of 2; for salivation of 3-4;
and for activity of 2-3). As a final note, no drug or drug combination resulted in alterations in ethanol drinking on subsequent days, even when cholinergic side effects were noted.
Discussion

The purpose of the present study was to characterize the efficacy of the AChR agonists xanomeline and varenicline in remediating a scopolamine-induced cognitive deficit in group-housed monkeys with ethanol self-administration histories. Subordinate monkeys, who experience chronic social stress took significantly more sessions to acquire the touchscreen training task. Despite this deficit and their higher lifetime ethanol intakes after one year of self-administration (Galbo-Thomma et al., 2023), subordinates did not differ from dominant monkeys in performance of the SDR task prior to the experiments with cholinergic drugs. Dominant and subordinate monkeys also did not differ in the potency of the muscarinic ACh antagonist, scopolamine, to produce a deficit in SDR performance, or in the effects of the agonists xanomeline or varenicline on SDR performance when the drugs were administered alone. However, both xanomeline and varenicline were effective in remediating a scopolamine-induced deficit, but only in subordinate monkeys. Importantly, these effects were observed at doses of scopolamine, xanomeline and varenicline that were not sedating or dysphoric, based on the low numbers of omissions observed and rarely observed side effects. It should also be noted that monkeys were not in a state of acute ethanol withdrawal, based on comprehensive observations in this cohort reported previously (Galbo-Thomma et al., 2023).

Based on slower rate at which they learned to use the touchscreens and higher lifetime ethanol intakes compared to dominant monkeys, it was expected that subordinate monkeys would perform worse on the SDR task. However, no rank-related differences were observed in the number of reversals completed, and these data were not correlated to lifetime ethanol intakes. It is possible that rank-related differences would have been observed if subordinate monkeys had higher lifetime intakes. Monkeys who begin self-administering ethanol during late adolescence and young adulthood have higher intakes during open access than monkeys who start drinking in middle adulthood (Helms et al., 2014). The present cohort started drinking ethanol around
age 12.5 (Galbo-Thomma et al., 2023). Had they started drinking at younger, they might have consumed more ethanol each day, leading to significantly poorer performance on the SDR task. Additionally, the initial assessment of performance on the SDR task consisted of only one session. Had monkeys performed the task repeatedly until meeting a pre-defined stability criterion, differences may have emerged. Moreover, although the SDR task used herein requires a relatively high cognitive demand (the cognitive capacity needed to solve a task), use of a more difficult cognitive task such as an ID/ED task, each session of which consists of 4 SDR components that increase in difficulty, may have produced rank-related differences. For example, in a group of ethanol-naïve, individually housed rhesus monkeys, initial performance on an ID/ED set-shifting task was identical between all monkeys, but across 30 sessions monkeys diverged into high- and low- performing groups (Shnitko et al., 2019). Because group differences were not observed in initial SDR performance in the present study, a deficit was induced with scopolamine reversal paradigm and the pro-cognitive effects of xanomeline and varenicline were assessed on that baseline.

As expected, we observed improved performance following vehicle administration between Experiments 1 and 2 due to repeated exposure to the task. However, this “practice” effect did not differ according to social rank. Nonetheless, because baseline SDR performance improved from Experiment 1 to Experiment 2, we determined scopolamine dose-effect curves and calculated ED$_{50}$ values in each experiment. Contrary to our hypothesis that subordinate monkeys would be more sensitive to the cognitive-disrupting effects of scopolamine, there was no rank-related difference in sensitivity to scopolamine. Neuroimaging studies in humans with heavy drinking histories indicated decreased brain mAChR expression (Freund & Ballinger, 1988, 1989; Hellström-Lindahl et al., 1993). As noted above, it is possible the lifetime intakes of the subordinate/heavier drinking monkeys in the present study were not high enough to produce sufficiently large changes in mAChR receptor distribution to alter SDR performance. However, a
more parsimonious explanation may be that the high affinity (~1-2 nM) of scopolamine for all mAChR subtypes masked group differences in mAChR receptors, leading to similar effects of the scopolamine on SDR performance in dominant and subordinate monkeys.

The M1/M4-preferring mAChR agonist xanomeline was more effective in remediating a scopolamine-induced deficit in cognitive flexibility in subordinate monkeys. This rank-related difference might be related to the higher lifetime ethanol intakes in subordinate monkeys. As noted above, human subjects with heavy alcohol drinking histories had decreased mAChR expression in various brain regions (Freund & Ballinger, 1988, 1989; Hellstrom-Lindahl et al., 1993). Importantly, two of these studies were conducted in histologically normal brains (i.e., no differences attributed to aging, medications, or other morbidities including dementia, head trauma or Alzheimer’s disease; Freund & Ballinger, 1988, 1989). More specifically, heavy drinking was associated with downregulation of M4 mAChR mRNA and protein (Walker et al., 2020); the effects of alcohol on M1 mAChR distribution have not been characterized (Walker et al., 2022). It is possible one year of ethanol self-administration decreased M1 and M4 mAChR receptor distribution in subordinate monkeys whereas dominant monkeys experienced little to no change. If so, M1 and M4 activation by xanomeline would increase ACh levels in subordinates, resulting in improved task performance. It is important to note, however, that rodent studies have indicated that mAChR subtype-specific function can fluctuate, even rebound, during alcohol consumption, withdrawal and abstinence (Walker et al., 2021). At the outset of the present studies, weekly ethanol access decreased from 5 days per week to 3-4 days per week to accommodate the testing schedule. Thus, mAChR distribution and function could have varied to some degree prior to our assessments of remediation.

Like xanomeline, the intermediate-efficacy α4β2/α7 nAChR agonist varenicline effectively improved scopolamine-disrupted performance in subordinates, but not in dominants. Little is
known about nAChR expression and distribution in individuals with heavy drinking histories. However, two NHP studies found reduced β2 subunit or α4β2 nAChR binding following 18 weeks or 6 months of ethanol self-administration, respectively (Cosgrove et al., 2010; Hillmer et al., 2014). The affinity of varenicline for the α4β2 nAChR is approximately 60% that of nicotine. Downregulation of nAChRs in subordinate/heavier drinking monkeys could result in increases in the efficacy of varenicline and cognitive enhancement (Buccafusco et al., 2005). In dominant/lighter drinking monkeys in whom nAChRs likely were not changed by ethanol, varenicline may function with lower efficacy, effectively antagonizing ACh and leading to a lack of engagement in the SDR task. The higher number of omissions made by dominant monkeys supports this hypothesis. Although such changes in mAChR and nAChR number and intrinsic efficacy were not directly measured in the present study, these findings suggest mAChR and nAChR agonists may be more effective in enhancing cognitive flexibility in individuals with heavy drinking histories. Although varenicline also acts as a low-affinity, high-efficacy agonist at α7 nAChRs, much less is known about the role of these receptors in cognition. It is thus more difficult to speculate about the consequences of potential ethanol-induced alterations of α7 nAChR (Childs et al., 2012). Future studies would be strengthened by incorporating measurements of AChR number, distribution, and intrinsic efficacy. They will also help to explain mechanisms underlying the observation that both XML and VAR were able to reverse the scopolamine-induced deficit in subordinate monkeys. Such an effect of XML might be expected based on the competition between XML and scopolamine to bind to mAChRs. The efficacy of VAR in dominant monkeys indicates that nAChR stimulation can overcome mAChR antagonism. Depending on exact changes in ACh receptors that occur in dominant and subordinate monkeys during chronic ethanol drinking, it is possible that multiple mechanisms may be targeted to result in cognitive improvements.
These studies are not without limitations. One is the absence of female subjects, which respond
to alcohol differently than males (Cameron, 1997; Carroll et al., 2015; Hayaki et al., 2020;
Kromrey et al., 2016; Mello, 1988). Moreover, we previously observed that menstrual cycle
phase affected both EtOH intakes under the present paradigm as well as performance of a SDR
task (Kromrey et al., 2015; Thomas & Czoty, 2019). Others have observed that heavy ethanol
self-administration can disrupt the menstrual cycle (e.g., Mello et al., 1988). Moreover, studies in
rodent indicated that there may be sex differences in basal ACh concentrations, cholinergic
signaling and receptor expression that are inherent or mediated by menstrual cycle phase
(Donny et al., 2000; Kirry et al., 2019; Koylu et al., 1997; Rhodes & Rubin, 1999; Witt et al.,
1986). Additionally, rank-related differences in cognitive flexibility may have pre-dated social
housing and/or ethanol exposure. For example, in female monkeys, poorer performance on a
working memory task predicted social rank (Kromrey et al., 2016). In addition, low cognitive
flexibility predicted future heavy drinkers in individually housed rhesus monkeys (Shnitko et al.,
2019). Thus, sex differences in the effects of alcohol and the ACh system caution against
extending these conclusions to female subjects.

Finally, we chose to use the muscarinic antagonist scopolamine to induce a cognitive deficit.
Although less invasive and disrupting than other methods of inducing a cognitive deficit such as
brain lesions, the approach is not without its limitations. A different method of inducing a
cognitive deficit, such as using a selective AChR antagonist or non-cholinergic drug, might have
generated group differences that were not observed in the present study. For example, NMDA
receptor antagonists, such as ketamine, dizocilpine (MK-801) and phencyclidine, are commonly
used to induce schizophrenia-like cognitive symptoms in preclinical research (Harder et al.,
1998; Rezvani, 2006). If there are social rank-related (or chronic ethanol intake-related)
differences in NMDA receptor number or function in NHPs, inducing a deficit with like these
might generate qualitative or quantitative differences on cognitive differences between dominant and subordinate monkeys that may be differentially amenable to pharmacotherapy.

In summary, the present studies characterized the effects of scopolamine on a serial SDR task in NHPs and the ability of the AChR agonists xanomeline and varenicline to improved cognitive flexibility in subordinate monkeys who had significantly higher lifetime ethanol intakes than dominant monkeys in this cohort. It is important for future studies to assess AChR number and intrinsic efficacy and to characterize these effects in females and subjects with more extensive ethanol self-administration histories.
Acknowledgements

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Data Availability Statement

The authors declare that all data supporting the findings are contained within the paper.
Authorship Contributions:

Participated in research design: Galbo-Thomma, Epperly, Saldaña, and Czoty

Conducted experiments: Galbo-Thomma

Contributed new reagents or analytic tools: Blough, Landavazo, and Carroll

Performed data analysis: Galbo-Thomma, and Czoty

Wrote or contributed to the writing of the manuscript: Galbo-Thomma, and Czoty
References


Brannan S, Sawchak S, Miller A, Paul SM, and Breier A (2020) Efficacy and safety of xanomeline, a M1/M4 receptor preferencing agonist, plus trospium, a peripheral muscarinic antagonist, in schizophrenia: Phase 2 clinical trial results. *Biological Psychiatry* **87:**S169.


Footnotes

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No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends.

**Figure 1.** The mean (± SD) number of sessions required for dominant (DOM) and subordinate (SUB) monkeys to acquire the stimulus fade training task (*p<0.05). Symbols depict individual subject data. Superscript indicates the pen (A,B,C) in which the monkeys resided.

**Figure 2.** The number of reversals acquired by dominant (DOM) and subordinate (SUB) monkeys in the SDR task and individual lifetime ethanol intakes after one year of ethanol self-administration.

**Figure 3.** Mean (± SD) number of reversals per session completed following administration of vehicle in Experiment 1 (Exp. 1) and Experiment 2 (Exp. 2) for dominant (DOM) and subordinate (SUB) monkeys (*p<0.05; ***p<0.001). Symbols represent individual subjects as in Fig. 1.

**Figure 4.** Mean (± SD) scopolamine ED$_{50}$ values in Experiments 1 (Exp. 1) and 2 (Exp. 2) for dominant (DOM) and subordinate (SUB) monkeys. Symbols represent individual subjects as described in Fig. 1.

**Figure 5.** Mean (± SEM) percent of trials as errors (left) and number of omissions made (right) after administration of vehicle (VEH) or the individually determined best dose of scopolamine (SCOP, filled bars) in Experiments 1 (Exp. 1) and 2 (Exp. 2) for dominant (DOM) and subordinate (SUB) monkeys (***p=0.001).

**Figure 6.** The mean (± SD) number of reversals acquired following administration of vehicle (S), xanomeline (XML, top row) or varenicline (VAR, bottom row) for individual dominant monkeys.
(D; left column) and subordinate monkeys (S; middle column). The right column shows mean (± SEM) number of reversals completed for dominant (DOM) and subordinate (SUB) monkeys. Because the highest doses were not tested in all animals, they were excluded from group analyses.

**Figure 7.** Mean (± SEM) percent remediation of a scopolamine-induced deficit by xanomeline (XML) and varenicline (VAR) in dominant (DOM) and subordinate (SUB) monkeys (**p<0.001**). Symbols represent individual subjects as described in Fig. 1.
Table 1. Scoring rubric for assessing the presence and severity of cholinergic side effects.

**Emesis**
0 – not present
1 – gagging but no emesis
2 – emesis present

**Salivation**
0 – not present
1 – slight salivation
2 – moderate, wet chin
3 – severe, face very wet
4 – (only documented at end of session) extremely severe, salivation persists through entire session

**Activity**
0 – normal
1 – inactive, compared to normal activity level; doesn’t reach for treat
2 – ataxic
3 – recumbent
Table 2. Effects of xanomeline (XML) and varenicline (VAR) in remediating cognitive deficits produced by scopolamine (SCOP) in dominant (D) and subordinate (S) monkeys. Superscript in first column indicates the pen (A,B,C) in which the monkeys resided.

<table>
<thead>
<tr>
<th>Monkey Rank-ID</th>
<th>Best dose combination (mg/kg)</th>
<th>SCOP, % change from VEH</th>
<th>XML+SCOP, % change from VEH</th>
<th>% Remediation</th>
<th>% Errors (± SD)</th>
<th>% Omissions (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-8491^A</td>
<td>0.1 XML + 0.017 SCOP</td>
<td>-70.0</td>
<td>-80.0</td>
<td>14.3</td>
<td>30.2 (3.7)</td>
<td>23.2 (13.5)</td>
</tr>
<tr>
<td>D-8490^A</td>
<td>0.1 XML + 0.03 SCOP</td>
<td>-56.5</td>
<td>-69.6</td>
<td>23.2</td>
<td>38.2 (9.4)</td>
<td>8.6 (6.8)</td>
</tr>
<tr>
<td>D-8493^B</td>
<td>0.3 XML + 0.03 SCOP</td>
<td>-91.3</td>
<td>-73.9</td>
<td>19.1</td>
<td>30.6</td>
<td>21.1</td>
</tr>
<tr>
<td>D-8501^B</td>
<td>0.03 XML + 0.017 SCOP</td>
<td>-63.2</td>
<td>-15.8</td>
<td>75.0</td>
<td>41.8 (4.3)</td>
<td>0.9 (0.1)</td>
</tr>
<tr>
<td>D-8494^C</td>
<td>0.03 XML + 0.03 SCOP</td>
<td>-100.0</td>
<td>-100.0</td>
<td>0.0</td>
<td>25.9</td>
<td>50.6</td>
</tr>
<tr>
<td>D-8108^C</td>
<td>0.03 XML + 0.01 SCOP</td>
<td>-71.4</td>
<td>-14.3</td>
<td>80.0</td>
<td>45.6 (2.6)</td>
<td>4.2 (0.4)</td>
</tr>
<tr>
<td>Mean (± SEM)</td>
<td>-75.4 (7.5)</td>
<td>-58.9 (15.9)</td>
<td>22.8 (20.0)</td>
<td>35.4 (3.4)</td>
<td>18.1 (8.2)</td>
<td></td>
</tr>
<tr>
<td>S-8497^A</td>
<td>1.0 XML + 0.017 SCOP</td>
<td>-58.3</td>
<td>-27.1</td>
<td>53.5</td>
<td>53.1 (2.2)</td>
<td>0.6 (0.5)</td>
</tr>
<tr>
<td>S-8496^A</td>
<td>0.1 XML + 0.01 SCOP</td>
<td>-79.5</td>
<td>-24.7</td>
<td>68.9</td>
<td>42.3 (2.64)</td>
<td>5.59 (0.5)</td>
</tr>
<tr>
<td>S-8528^B</td>
<td>0.1 XML + 0.03 SCOP</td>
<td>-94.8</td>
<td>-47.9</td>
<td>49.5</td>
<td>46.3 (3.5)</td>
<td>0.3 (0.4)</td>
</tr>
<tr>
<td>S-8527^B</td>
<td>0.03 XML + 0.03 SCOP</td>
<td>-78.1</td>
<td>-38.4</td>
<td>50.8</td>
<td>56.2 (2.5)</td>
<td>0.6 (0.2)</td>
</tr>
<tr>
<td>S-8495^C</td>
<td>0.3 XML + 0.03 SCOP</td>
<td>-93.5</td>
<td>-54.2</td>
<td>42.0</td>
<td>51.0 (2.0)</td>
<td>0.1 (0.2)</td>
</tr>
<tr>
<td>S-8500^C</td>
<td>0.3 XML + 0.017 SCOP</td>
<td>-89.6</td>
<td>-27.1</td>
<td>69.8</td>
<td>48.0 (5.1)</td>
<td>2.1 (0.2)</td>
</tr>
<tr>
<td>Mean (± SEM)</td>
<td>-82.3 (6.1)</td>
<td>-36.6 (5.5)</td>
<td>55.8 (5.0)</td>
<td>49.5 (2.2)</td>
<td>1.6 (0.9)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Monkey Rank-ID</th>
<th>Best dose combination (mg/kg)</th>
<th>SCOP, % change from VEH</th>
<th>VAR+SCOP, % change from VEH</th>
<th>% Remediation</th>
<th>% Errors (± SD)</th>
<th>% Omissions (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-8491^A</td>
<td>0.1 VAR + 0.017 SCOP</td>
<td>-77.5</td>
<td>-71.5</td>
<td>7.7</td>
<td>40.4 (4.3)</td>
<td>6.6 (3.8)</td>
</tr>
<tr>
<td>D-8490^A</td>
<td>0.01 VAR + 0.03 SCOP</td>
<td>-86.2</td>
<td>-75.9</td>
<td>11.9</td>
<td>36.7 (0.3)</td>
<td>14.1 (0.4)</td>
</tr>
<tr>
<td>D-8493^B</td>
<td>0.1 VAR + 0.03 SCOP</td>
<td>-94.4</td>
<td>-75.0</td>
<td>20.6</td>
<td>46.6 (2.8)</td>
<td>1.6 (1.2)</td>
</tr>
<tr>
<td>D-8501^B</td>
<td>0.03 VAR +</td>
<td>-60.9</td>
<td>-73.9</td>
<td>-21.3</td>
<td>43.7 (3.5)</td>
<td>0.3 (0.2)</td>
</tr>
</tbody>
</table>
Table 3. Total number of monkeys who displayed more severe cholinergic side effects for various drug doses or combinations.

<table>
<thead>
<tr>
<th></th>
<th>Emesis</th>
<th>Salivation</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present</td>
<td>Severe</td>
<td>Extremely severe</td>
</tr>
<tr>
<td>0.03 SCOP</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.056 SCOP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.3 XML</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>1.0 XML</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>0.3 VAR</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>0.17 VAR</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.01 VAR + 0.03 SCOP</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.17 VAR + 0.03 SCOP</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 2

Table: Intake (g/kg)

<table>
<thead>
<tr>
<th>DOM</th>
<th>Intake (g/kg)</th>
<th>SUB</th>
<th>Intake (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-8491A</td>
<td>223.60</td>
<td>S-8497A</td>
<td>694.11</td>
</tr>
<tr>
<td>D-8490A</td>
<td>207.97</td>
<td>S-8496A</td>
<td>550.51</td>
</tr>
<tr>
<td>D-8493B</td>
<td>329.98</td>
<td>S-8528B</td>
<td>369.43</td>
</tr>
<tr>
<td>D-8501B</td>
<td>521.61</td>
<td>S-8527B</td>
<td>294.17</td>
</tr>
<tr>
<td>D-8494C</td>
<td>103.94</td>
<td>S-8495C</td>
<td>378.57</td>
</tr>
<tr>
<td>D-8108C</td>
<td>195.89</td>
<td>S-8500C</td>
<td>286.71</td>
</tr>
</tbody>
</table>
Figure 3
Figure 4
**Figure 5**

Bar charts showing the mean percent of errors and omissions for two experiments (Exp. 1 and Exp. 2) for two groups: VEH (white bars) and SCOP (gray bars).

- **Errors**
  - Exp. 1: DOM 50%, SUB 40%
  - Exp. 2: DOM 40%, SUB 30%

- **Omissions**
  - Exp. 1: DOM 10%, SUB 8%
  - Exp. 2: DOM 7%, SUB 6%

Significance: ***p < 0.001
Figure 6
Cognitive-enhancing effects of acetylcholine receptor agonists in group-housed cynomolgus monkeys who drink ethanol

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Supplementary Figure 1. The individual dose-effect curves for each subordinate (S) monkey for vehicle (0.0), scopolamine (SCOP; filled symbols) and xanomeline (XML; open symbols).

Supplementary Figure 2. The individual dose-effect curves for each dominant (D) monkey for vehicle (0.0), scopolamine (SCOP; filled symbols) and xanomeline (XML; open symbols).
Supplementary Figure 3. The individual dose-effect curves for each subordinate (S) monkey for vehicle (0.0), scopolamine (SCOP; filled symbols) and varenicline (VAR; open symbols).

Supplementary Figure 4. The individual dose-effect curves for each dominant (D) monkey for vehicle (0.0), scopolamine (SCOP; filled symbols) and varenicline (VAR; open symbols).