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# Novel Antimuscarinic Antidepressant-like Compounds with Reduced Effects on Cognition

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List of non-standard abbreviations: L-687,306, 5-((1R,3R,4R)-1-azabicyclo[2.2.1]heptan-3-yl)-3-cyclopropyl-1,2,4-oxadiazole; CJ2100, 3-cyclopropyl-5-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,4-oxadiazole oxalate; p-tosyl, p-toluenesulfonic acid; EtOH, ethanol; NMDA, N methyl D-aspartate; AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; MDD: Major depressive disorder; SSRI: Selective serotonin reuptake inhibitor

## ABSTRACT

The cholinergic nervous system has been implicated in mood disorders, evident in the fast-onset antidepressant effects of scopolamine, a potent muscarinic antagonist, in clinical studies. One prominent disadvantage to the use of scopolamine in the treatment of depression is its detrimental effects on cognition, especially as such effects might aggravate cognitive deficits that occur with depression itself. Thus, the identification of antimuscarinic drugs that are free of such detrimental effects may provide an important avenue for the development of novel therapeutics for the management of depression. The present data in rats indicate that a historical muscarinic antagonist, L-687,306, and a muscarinic antagonist of our own design, CJ2100, were as or more effective than scopolamine in antagonizing both the bradycardic effects of the muscarinic agonist arecoline in cardiovascular studies and its discriminative-stimulus and rate-decreasing effects in behavioral studies. Additionally, both novel muscarinic antagonists were as effective as scopolamine in decreasing immobility in the forced swim test (FST), a preclinical indicator of potential antidepressant activity. However, at equi-effective or even larger doses, they were considerably less disruptive than scopolamine in assays of cognition-related behavior. All three drugs displayed high specificity for the mAChRs with few off-target binding sites, and CJ2100 showed modest affinity across the mAChRs when compared to L-687,306 and scopolamine. These data emphasize the dissimilar pharmacological profiles that are evident across antimuscarinic compounds and the potential utility of novel antagonists for the improved treatment of depression.

## **SIGNIFICANCE STATEMENT**

Some clinical studies with the muscarinic antagonist scopolamine document its ability to produce antidepressant effects in patients with mood disorders; however, scopolamine also has well-known adverse effects on both autonomic and centrally-mediated physiological functions that limit its therapeutic use. Here, we characterize the cardiovascular and discriminative stimulus effects of two novel muscarinic antagonists, L-687,306 and CJ2100, that produce antidepressant-like effects in a rodent model (forced swim test), without affecting touchscreen-based cognitive performance (titrating vigilance and delayed matching-to-position).

## INTRODUCTION

Major depressive disorder (MDD) affects approximately 16% of Americans (Kessler et al. 2006) and, notwithstanding some recent advances, an urgent need remains for antidepressant drugs that, unlike tricyclic antidepressants and selective serotonin reuptake inhibitors, have immediate beneficial effects. Furthermore, many currently available antidepressant medications impair cognition and produce other adverse effects that limit their widespread application (Blair and Dauner, 1992; Sayyah et al. 2016). Improvement in side-effect liability, as well as faster onset and better clinical efficacy, are major therapeutic goals in the development of novel antidepressants (O'Leary et al., 2015; Wang et al., 2015).

It is widely accepted that the cholinergic nervous system plays a prominent role in learning and memory. It is becoming better known that this system is also implicated in the control of mood states (Leaderbrand et al., 2016; Drevets et al., 2020; Dulawa and Janowsky, 2019; Deutsch, 1971). For example, clinical data show that the muscarinic AChR (mAChR) antagonist scopolamine produces antidepressant effects in patients with either MDD or bipolar disorder (Drevets and Furey, 2010; Drevets et al., 2013; Drevets et al., 2020; Furey et al., 2006; Witkin et al., 2020). Despite such intriguing and hopeful clinical findings, the therapeutic value of scopolamine for treating MDD is limited by its well-documented adverse effects, which include disturbances that are both autonomic (blurred vision, tachycardia) and centrally-mediated (drowsiness, light-headedness) (Drevets et al., 2020). Large doses, potentially above those required for antidepressant activity, can result in mental confusion or delirium, and memory loss) (Drevets et al., 2020); Furey et al., 2008; Lakstygall et al., 2019; Klinkenberg and

Blokland, 2010). Since MDD is also associated with deficits in cognition (Porter et al., 2003; Clark et al., 2009), the use of scopolamine has the potential to exacerbate this problem. Nevertheless, the favorable clinical data obtained with scopolamine have led to the question of whether it may be possible to develop antimuscarinic drugs that act via anticholinergic mechanisms to relieve depression, yet have fewer effects on learning, memory, and attention.

One compound that has spurred interest in its further development as an antidepressant medication is L-687,306. This novel muscarinic ligand, developed as a possible treatment for Alzheimer's disease in the early 1990s (Freedman et al., 1992; Baker et al., 1992; Freedman, et al., 1993), was shown to have good affinity with limited or no efficacy at the M<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub> receptor subtypes and, in vivo, a reduced side-effect profile compared to conventional antimuscarinics like scopolamine. L-687,306 was shown to attenuate the brief, but profound bradycardia produced by the muscarinic agonist arecoline (Winger et al., 2020), indicative of its scopolamine-like antagonist actions in the autonomic nervous system. L-687,306 was able to antagonize both the discriminative-stimulus and rate-suppressing effects of arecoline far better than scopolamine (Winger et al., 2020), confirming that it has central anticholinergic activity with apparently less rate suppressing, possibly sedative, effects compared with scopolamine. In conjunction, these findings encourage the view that it is possible to develop novel antimuscarinics that may be therapeutically effective and produce fewer side effects than the conventional antimuscarinic scopolamine. Notwithstanding these intriguing findings with L-687,306, its complicated stereoselective synthesis has made it a difficult compound to study. To address this limitation, we have recently advanced a

cyclopropyl analogue of arecoline, CJ2100. Of importance to our efforts, CJ2100, unlike L-687,306, can be produced in only a few synthetic steps with readily available starting materials.

The purpose of the present studies was to compare the activity of L-687,306 and CJ2100 with that of scopolamine *in vitro* and across several *in vivo* assays of autonomic and central activity. The autonomic effects of the three antimuscarinics (scopolamine, L-687,306 and CJ2100) were interrogated by examining their ability to antagonize the effects of arecoline on heart rate. The centrally-mediated effects of these antimuscarinics were studied by determining their ability to a) antagonize the discriminative stimulus and rate suppressing effects of arecoline; b) decrease measures of immobility in the forced swim test (FST) as a first-pass indication of potential antidepressant action; c) produce a direct disruption of cognition-related behavior. The outcome of these studies shows that we have identified molecules that bind with high specificity to the mAChRs with established antimuscarinic actions and scopolamine-like activity in the FST, but with few other direct behavioral effects. These findings show that disruption of cognition-related behavior is not an inevitable result of blockade of muscarinic receptors and, thus, support the further development of antidepressant drugs with an antimuscarinic basis of action and reduced side-effect liability.

## METHODS

### *Animals*

Adult male Sprague-Dawley rats weighing approximately 275-375 grams purchased from Envigo RMS (Indianapolis, IN) were used in the cardiovascular,

arecoline discrimination, and FST studies. Due to superior visual abilities in rat strains with pigmented eyes (Jacobs et al., 2001), 12 adult Long-Evans rats (6 male and 6 female) weighing approximately 275-375 grams purchased from Charles River Laboratories (Wilmington, MA) were used in the touchscreen-based cognition studies. Previous work demonstrated that there were few, if any, behavioral differences or effects of drugs in the FST between Sprague-Dawley and Long-Evan rats (Jutkiewicz et al., 2003). All rats were housed in climate-controlled vivaria with a 12 h light/dark cycle with lights on at 7:00 AM. Subjects in the cardiovascular assay and FST had ad libitum access to food and water. All other animals were maintained at approximately 85% of their free-feeding weight via post-session portions of ~10-15 grams of rodent chow and had unrestricted access to water in their home cage. Experimental sessions were conducted 5 days a week (Mon-Fri). All experimental protocols were approved by the Institutional Animal Care and Use Committees at the University of Texas Health San Antonio, the University of Michigan, and McLean Hospital, and conformed to the guidelines from the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, Commission on Life Sciences (National Research Council, 2011).

#### *In vitro Assays:*

Primary and Secondary Binding Assays were performed by the National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH PDSP). The NIMH utilizes an integrated sample storage, retrieval and liquid handling robotic system to prepare assay plates. Using stable CHO lines expressing one of M<sub>1</sub>-M<sub>5</sub> mAChRs,

primary radioligand binding assays are performed using a 96-well drug plate containing multiple test compounds (each run in quadruplicate), one reference compound, or buffer only ( $[^3\text{H}]\text{QNB}$  only to determine total binding) with 25  $\mu\text{l}$  per well at 50  $\mu\text{M}$ .

From primary assays, compounds showing a minimum of 50% inhibition at 10 $\mu\text{M}$  are evaluated in secondary radioligand binding assays to determine binding affinities at equilibrium for specific targets of interest. Compounds are tested at 11 concentrations in triplicate with the hot ligand at concentrations close to its  $K_d$ . Readers are referred to the procedures found on the PDSP website for specific experimental details:

<https://pdsp.unc.edu/ims/investigator/web/>.

### *Cardiovascular assay*

Rats were prepared with indwelling transmitters to obtain heart rate data. Under ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia, a telemetric transmitter (TA11PA-C40 or TL11M2-C50-PXT, Data Sciences International, Transoma Medical Inc., St. Paul, MN, USA) was surgically inserted into a subcutaneous pocket on the rat's side. Transmitters conveyed heart rate data that was collected and stored digitally on computer using Physiotel receivers and the DSI Data Exchange Matrix (Data Sciences International, Transoma Medical Inc., St. Paul, MN, USA), and compiled with the Dataquest A.R.T. system and its Gold Analysis 3.01 software. Following surgery, rats were singly housed and allowed to recover for at least 7 days before initiating studies. The procedures are described in detail in (Jutkiewicz et al., 2013), and followed the description of cardiovascular assays by (Delaunois et al., 2009). At the start of an experimental session, rats in their home cages were placed on top of the receivers;

heart rate data were collected for at least 1 h from undisturbed animals. Each rat was then given a saline injection to provide data on the response to injection and handling procedures. Thirty min later a second injection was given either a second saline injection, 0.3 mg/kg CJ2100, or 1 mg/kg CJ2100. Fifteen min later, 10 mg/kg arecoline was given as the final injection. Heart rate measures were taken during the light cycle, between 10:00 AM and 3:00 PM. Data were collected for at least 2 h following the last injection. All injections were administered subcutaneously (s.c.) in a volume of 1 ml/kg.

This same procedure was used to evaluate the effects of a range of doses of L-687,306 and scopolamine on arecoline-induced bradycardia. Those data are published in Winger et al (2020) and the most effective doses of these antagonists are included in the results section for comparison to CJ 2100.

### *Arecoline Discrimination*

Drug discrimination procedures were performed in standard 2-lever operant conditioning chambers (30.5×24.1×21.0 cm) with stainless steel grid floors and contained within ventilated, sound-attenuating boxes (Med Associates, St. Albans, VT). Subjects (male Sprague-Dawley rats) were initially trained to respond under a fixed-ratio (FR) 10 schedule of reinforcement on either lever. Completion of the schedule requirements resulted in delivery of a 45 mg sucrose pellet. Subsequently, on alternating days, saline or arecoline (1 mg/kg) was given s.c. just before the rat was placed in the chamber. The session began 5 min later with illumination of the house light and stimulus lights illuminated and presentation of both levers. Responses on the left lever were designated “correct” following drug administration; completing the FR

requirement on that lever resulted in retraction of both levers and delivery of a sucrose pellet. When saline rather than arecoline was injected before a session, completing the FR requirement on the right lever resulted in retraction of the levers and reinforcer delivery. Completing the FR requirement on the incorrect lever caused the levers to be retracted, and the stimulus lights to be turned off for 10 sec with no sucrose pellet delivery. Testing began when the following criteria were met on two consecutive training days: 1) responding on the first FR of the session was completed on the injection-appropriate lever, and 2) >85% of total session responses were made on the injection-appropriate lever. During test sessions, completed FRs on either lever were reinforced with sucrose pellet delivery. For evaluation of antagonist effects, a dose of the selected antagonist was administered 15 min before the start of the session, and animals were returned to their home cages. Arecoline was given immediately before the rats were placed in the response chambers, as indicated above. Rats were tested no more than twice weekly.

### *Forced Swim Test (FST)*

The FST used in the current study has been described previously (Jutkiewicz et al., 2003; 2004) and consists of a one-day, 15 min swim only. Previous studies demonstrated that known, clinically used antidepressants produce similar effects in a one-day FST to those observed in the 2-day Porsolt swim test (Broom et al., 2002). Removing the first “pre-exposure” swim may eliminate some concerns about rats “learning” to become immobile and the potential effects of evaluating drugs that alter learning and memory (e.g., scopolamine). While the forced swim test lacks face- and

construct-validity for modeling depression in rats or mice, this assay is useful for identifying drugs that have antidepressant actions in clinical populations (for reviews see Borsini and Meli, 1988; West, 1990; Lucki, 1997; Petit-Demouliere et al., 2005; Abelaira et al., 2013; Commons et al., 2017; Unal and Canbeyli, 2019).

In the current study, rats (male Sprague-Dawley) were placed in a cylindrical container (46 cm tall x 20 cm diameter) that contained 25° C water to a depth of 30 cm. Test compounds were given by subcutaneous injection 15 min prior to the swim period ( $n=6$  rats per dose per drug). Rats swam for 15 min and behavior was videotaped from above the swim tank for the entire swim duration. The videotapes were scored for swimming (moving limbs underwater in an active manner), climbing (active forepaw movement out of the water, usually against the side of the chamber), and immobility (floating with sufficient movement only to keep the head above water) by a trained observer who was blinded to the testing condition (Broom et al., 2002). Behavior was recorded every 5 sec over the 15 min swim period. Following the swim session, rats were removed from the water, towed-dried, and placed in a warmed cage for 10-15 min.

### *Locomotor Activity*

Locomotor activity was measured in square, acrylic arena (14" X 14" X 8") with infrared beams spaced 2 inch apart. Infrared detectors were placed along both sides of the arena (X and Y axes) 1 in off the arena floor to capture ambulation and along one side of the arena (X axis) 6 in off the arena floor to capture rearing behaviors (Opto-M3 Activity Monitor). Beam breaks were quantified using the Multi-Device Interface

Software (Columbus Instruments, Columbus, OH). Male Sprague-Dawley rats (N=8 per condition) were habituated to the arena for at least 1 hr and then received a subcutaneous injection of saline. Approximately 30 min later, rats received an injection (SC) of saline, 0.3 mg/kg scopolamine, 3 mg/kg L687,306, or 3 mg/kg CJ2100, and beam breaks were recorded for at least 3 hours.

### *Titration Delay Matching-to-Position*

Studies of cognition-related behavior were conducted in touch-sensitive experimental chambers custom designed for rats (Kangas and Bergman, 2017). A titrating delay matching-to-position task (modified for rats from Kangas et al., 2010) was used to examine the effects of scopolamine and other muscarinic antagonists on short-term spatial memory. Each trial began with presentation of a 5x5 cm sample stimulus (green box) on the 17" touchscreen (black background) in one of two screen locations (to the far left or far right of center). The subjects (male and female Long-Evans rats, selected for their good eyesight) were required to touch the stimulus 3 times (FR3). Following completion of this response requirement, the retention interval began. The stimulus disappeared and was replaced by a 5x5 cm purple circle that was centered near the top of the touchscreen, requiring the subject to rear upward to respond. Requiring a response to this center stimulus positioned the subject in the center of the chamber during the retention interval. The first response to the center stimulus after the retention interval elapsed resulted in the removal of the center stimulus and presentation of both the left and right comparison stimuli. A response to the comparison stimulus on the side that matched the previously presented sample stimulus resulted in

a 0.1 ml milk reward paired with an 880 msec 440Hz tone and yellow screen flash that was followed by a 10 sec blackout intertrial interval. An incorrect response resulted in a 20 sec blackout intertrial interval. The initial retention interval was 0-sec during the first trial of each 48-trial session. If the subject correctly matched the previously presented stimulus position on two consecutive trials, the retention interval on the following trial increased by 1 sec; each error decreased the retention interval by 1 sec. These titrating contingencies were designed to capture the subject's short-term spatial memory across 48-trial training sessions. Steady-state performance in individual subjects was obtained prior to drug testing and was defined as 5 consecutive session means within  $\pm 20\%$  of the average mean of the 5 sessions. Subjects were given doses of scopolamine (0.01-3.2 mg/kg), L-687,306 (0.1-10 mg/kg), or CJ2100 (0.1-10 mg/kg), or saline 15 min prior to select test sessions with at least 3 intervening training sessions.

### *Psychomotor Vigilance Task*

A psychomotor vigilance task modified for rats from (Mackworth, 1948; Kangas et al., 2016; Weed and Gold, 1998) was used to examine the effects of scopolamine and other muscarinic antagonists on attentional processes. Each trial began with presentation of a 7x7 cm stimulus (pink square) on the screen (blue background) in one of six locations (evenly spaced 3x2 matrix). The intermittency of stimulus presentation (after a 15-, 30-, or 45- sec intertrial interval) as well as the stimulus location were randomized across trials. The duration of stimulus presentation was 2 sec on the first trial. If the subject successfully responded by rearing and touching the stimulus with its paw within the 2 sec duration, 0.1 ml milk reward was delivered paired with an 880

msec 440Hz tone and yellow screen flash, and the stimulus duration on the following trial was decreased by 0.25 sec. If the subject failed to respond to the stimulus before the duration had elapsed, the duration of the stimulus increased by 0.25 sec on the following trial. These titrating contingencies were designed to capture the subject's reaction time and ability to maintain task performance across a 96-trial training session (i.e., focused vigilance). Steady-state performance in individual subjects was obtained prior to drug testing and was defined as 5 consecutive session means within  $\pm 20\%$  of the average mean of the 5 sessions. Subjects were given scopolamine (0.01-3.2 mg/kg), L-687,306 (0.1-10 mg/kg), or CJ2100 (0.1-10 mg/kg), or saline 15 min prior to select test sessions with at least 3 intervening training sessions.

## DATA ANALYSIS

*Cardiovascular Assay.* The analysis program calculated an average heart rate every 10 sec. These 10 sec epochs were averaged over 1 min per rat, and data from 4 to 8 rats were averaged for each treatment group with standard error of the mean as a measure of variability. The graphs show data for 20 min following administration of arecoline. Data were fitted with a linear mixed model (jamovi version 1.2) with  $p < 0.001$  as criteria for significance.

*Arecoline Discrimination.* The completion of at least one FR was required for discrimination analysis. Full or complete generalization to a discriminative cue was defined as  $>85\%$  of responding on the drug-associated aperture. Response rate was calculated as number of responses made on either lever during the period when the stimulus light was illuminated.

*Forced Swim Test (FST).* The total counts for each behavior were averaged across rats within each treatment group. Data were analyzed by one-way analysis of variance (ANOVA). When appropriate, ANOVAs were followed by a Dunnett's post hoc analysis. The criterion for significance was set at  $p < 0.05$ . All statistical analyses were conducted using GraphPad Prism 8 Software (San Diego, CA, USA).

*Locomotor activity.* The number of consecutive beam breaks in the X-Y plane and the Z plane were recorded every minute, added together, and then summed into 10 min bins. Beam breaks were averaged within a treatment group to generate a time course of activity. Data were analyzed by repeated measure two-way ANOVA with time (repeated measure, within subject) and dose (between subject) as independent variables. Since a significant interaction was not expected, main effects between treatments were compared with Tukey's multiple comparisons post-hoc analyses. The criterion for significance was set at  $p < 0.05$ . All statistical analyses were conducted using GraphPad Prism 8 Software (San Diego, CA, USA).

*Titrating Delay Matching-to-Position Task.* The primary dependent measure under training conditions was mean titrated retention interval across the 48 trials.

*Psychomotor Vigilance Task.* The primary dependent measure under training conditions was mean titrated duration value across the 96 trials.

Data from the FST, titrating delay matching-to-position task, and psychomotor vigilance tasks were analyzed by one-way analysis of variance (ANOVA). When appropriate, ANOVAs were followed by a Dunnett's post hoc analysis. The criterion for

significance was set at  $p < 0.05$ . All statistical analyses were conducted using GraphPad Prism 8 Software (San Diego, CA, USA).

## DRUGS

Scopolamine hydrobromide trihydrate was purchased from Sigma Aldrich (St. Louis, MO). L-687,306 and CJ2100 were synthesized for these studies by the present author (CRJ) in the Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy.

<insert Figure 1 here>

### *Synthesis of L 687,306*

The procedure of (Cottrell et al., 1991) was modified for the synthesis of L687,306 (Figure 1). An acid catalyzed [2+3] cycloaddition with azomethine ylide precursor (2) and 5,6-dihydropyranone afforded the pyrrolidine ring system as a 1:1 mixture of diastereomers that was purified via the formation of the maleate salt. The mixture (free-base) was then saturated with HBr (acetyl bromide/ethanol) which resulted in the fracture of the pyranone ring to yield (5) and (6), which were neutralized with sodium carbonate to afford a mixture of 3*S*,4*R* and 3*R*,4*S* quaternary salts. Recrystallization in acetone/diethyl ether afforded the pure (*R*) diastereomer (7). Transfer hydrogenation with 1,4-cyclohexadiene to remove the chiral auxiliary afforded (10) which was recrystallized in acetone/diethyl ether to provide the hydrobromide salt. After free basing via standard protocol, oxadiazole formation with *N*'-hydroxycyclopropanecarboximidamide with sodium hydride under reflux yielded the desired compound. This method was preferred to that over previous work by Street et

al., 1990) as it allowed scalable access to the desired compound in enantiomerically pure form.

### **5-((1R,3R,4R)-1-azabicyclo[2.2.1]heptan-3-yl)-3-cyclopropyl-1,2,4-**

**oxadiazole (L-687,306):** The p-tosyl salt was generated according to literature procedures (Houghton et al., 1993). MS (ESI<sup>+</sup>): C<sub>11</sub>H<sub>15</sub>N<sub>3</sub>O, 206.1 (M+H<sup>+</sup>). Optical rotation was measured of intermediate (8) as detailed in (Cottrell et al., 1991): [α]<sub>D</sub> +29.7° (c=0.5 in EtOH).

<insert Figure 2 here>

### *Synthesis of CJ2100*

The procedure of (Street et al., 1990; Showell et al., 1991) was modified for the synthesis of CJ2100 (Figure 2). Arecoline was free based and reacted with cyclopropyl oxime under basic conditions to afford the oxime in moderate yield. Subsequent oxalate salt formation followed by recrystallization (EtOH/diethyl ether) afforded CJ2100 as a white solid.

### **3-cyclopropyl-5-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,4-oxadiazole oxalate**

**(CJ2100):** <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400 MHz, ppm): δ 7.07-7.05 (m, 1H), 3.33-3.30 (m, 2H), 3.18-3.17 (m, 2H), 2.89 (s, 3H), 2.65-2.63 (m, 2H), 2.00-1.96 (m, 1H), 0.97-0.88 (m, 2H), 0.87-0.85 (m, 2H). <sup>13</sup>C NMR (CD<sub>3</sub>OD, 100 MHz, ppm): δ 171.02, 170.18, 132.58, 116.35, 48.47, 47.62, 40.19, 21.19, 5.05, 4.31. MS (ESI<sup>+</sup>): C<sub>11</sub>H<sub>15</sub>N<sub>3</sub>O, 206.1 (M+H<sup>+</sup>).

## RESULTS

### *In vitro Binding Assays*

<insert Table 1>

Table 1 shows the binding profile of L-687,306, CJ2100, and scopolamine. As shown in Table 1, L-687,306, CJ2100, and scopolamine have a high specificity for the mAChRs, with little off-target binding. L-687,306 displays 2-5 fold higher affinity for the individual mAChR subtypes than CJ2100, but still 10-100 fold lower affinity compared with scopolamine. This correlates well with *in vivo* doses between the two compounds as shown in the following figures. The only notable off target binding site is CJ2100 at 5HT<sub>2B</sub>, although follow-up efficacy screens are necessary to determine if this is pharmacologically significant.

### *Heart Rate*

<insert Figure 3>

Figure 3 shows the dramatic and short-lived effect of s.c.10 mg/kg arecoline on heart rate. A dose of 0.3 mg/kg CJ 2100 produced some attenuation of the arecoline-induced bradycardia, and a dose of 1.0 mg/kg CJ2100 was completely effective in blocking this effect of arecoline. Figure 3 includes data on the smallest doses of L 687,306 and scopolamine that prevented arecoline's agonist effects on heart rate. Complete dose-effect curves using these two muscarinic antagonists are shown in Winger et al (2020); these data are included in the figure for comparison to CJ 2100. The effects of treatment ( $F_{[4,180]}=57.80$ ), time ( $F_{[9,216]}=20.1$ ), and the treatment x time interaction ( $F_{[36,216]}=8.11$ ) were each statistically significant ( $p<0.001$ ).

Bonferroni-corrected multiple comparisons among the cells means that the results obtained with arecoline were significantly different from saline at 2-5 min only ( $p < 0.001$ ). Each of the three muscarinic antagonists given 15 min earlier largely prevented this arecoline-induced bradycardia.

These data indicate that these doses of the antimuscarinic compounds are equally effective in antagonizing arecoline-induced bradycardia. In conjunction with previous data on scopolamine and L-687,306 under identical conditions (Winger et al., 2020), these findings indicate that scopolamine is 10 times more potent than L-687,306, and CJ2100 is likely to be at least equipotent with L-687,306 in this measure of antimuscarinic action.

#### *Arecoline Discrimination*

<insert Figure 4>

As shown in Figure 4A, scopolamine was unable to greatly modify the discriminative stimulus effects of arecoline (top) at doses that, in combination with arecoline, produced profound decreases in rates of responding (bottom). L-687,306 (Figure 4B), on the other hand, produced a dose-related reduction in the potency of arecoline as a discriminative stimulus (top). L-687,306 also markedly decreased the ability of arecoline to suppress rates of responding (bottom). CJ2100 (Figure 4C), similar to L-687,306, attenuated both the discriminative stimulus effects of arecoline (top) and the rate-suppressing effects of arecoline (bottom), although this effect was not as dose-related or as profound as that shown with L-687,306.

### *Forced Swim Test*

<insert Figure 5>

As shown in Figure 5A, scopolamine produced a dose-dependent decrease in immobility ( $F(3,20)=5.5$ ,  $p=0.007$ ) following administration of 0.1 mg/kg ( $p<0.05$ ) and 0.3 mg/kg ( $p<0.01$ ). Also, scopolamine increased swimming behaviors ( $F(3,20)=3.5$ ,  $p=0.04$ ) with a significant increase observed following administration of 0.3 mg/kg ( $p<0.05$ ). Although scopolamine slightly increased climbing behavior at 0.1 mg/kg, these effects were not statistically significant ( $F(3,20)=2.6$ ,  $p=0.08$ ). L-687,306 dose dependently decreased immobility ( $F(3,20)=12.5$ ,  $p<0.0001$ ), increased swimming ( $F(3,20)=3.4$ ,  $p=0.04$ ), and increased climbing behaviors ( $F(3,20)=4.7$ ,  $p=0.01$ ), but all of these effects were statistically significant only at the 3 mg/kg dose (Figure 5B). CJ2100 decreased immobility ( $F(3,20)=7.9$ ,  $p=0.001$ ) and increased swimming behaviors ( $F(3,20)=7.6$ ,  $p=0.001$ ) at the largest dose tested (3 mg/kg). However, CJ2100 did not alter climbing behaviors at any dose tested (Figure 5C).

### *Locomotor activity*

The effects of scopolamine (0.3 mg/kg), L687,306 (3 mg/kg), and CJ2100 (3 mg/kg) on locomotor activity were evaluated at doses that produced significant decreases in immobility in the FST (Figure 5D). While there was no significant interaction of treatment and time as expected, there were significant main effects of treatment [ $F(3,28) = 6.6$ ,  $P=0.002$ ] and time [ $F(10,280)=31.6$ ,  $P<0.0001$ ]. Injection, handling, and saline injection increased locomotor activity initially that returned to baseline (BL) levels within 20-30 min. Similar to the saline condition, injection of

scopolamine, L687,306, and CJ2100 all increased locomotor activity immediately after injection, but only L687,306 significantly increased locomotor activity as compared with the saline injection (Tukey's posthoc analysis of main effects of treatment,  $P=0.001$ ). The doses of scopolamine and CJ2100 tested did not significantly alter locomotor activity in this assay.

### *Psychomotor Vigilance Task*

<insert Figures 6 and 7>

As shown in the of Figure 6A, under control and saline conditions, all male and female rats consistently responded to the presented stimulus within the 2 sec duration of its presentation. Scopolamine produced significant dose-related adverse effects on mean titrated duration relative to performance following saline administration ( $F(5)=9.53$ ,  $p<0.0001$ ). Small doses of scopolamine (0.01-0.1 mg/kg) somewhat increased the variability of the responding, but did not significantly increase the mean titrated duration of the stimulus. Larger doses of scopolamine, however, significantly increased the mean titrated duration of the stimulus following administration of 0.32 ( $p<0.01$ ), 0.1 ( $p<0.001$ ), and 3.2 mg/kg ( $p<0.001$ ). Administration of L-687,306 failed to impair performance under this task across a range of doses (0.1-10 mg/kg). Similarly, a wide range of doses of CJ2100 (0.1-10 mg/kg) did not modify the ability of the rats to respond in this task; mean titrated duration of the stimulus remained at approximately 2 sec with very little variability.

### *Titration Delay Matching-to-Position*

As shown in Figure 6B, under control conditions and following saline administration, titrating delay matching-to-position performance consistently produced mean titrated delay values approximating 7 sec with low levels of variability among the male and female rats. Scopolamine treatment was associated with significant dose-related impairment of spatial memory relative to performance following saline treatment ( $F(5)=7.52$ ,  $p=0.0007$ ), which is reflected by decreases in accuracy resulting in lower mean titrated duration values, most evident following administration of 0.1 and 0.32 mg/kg ( $p<0.001$ ). Unlike scopolamine, however, both L-687,306 ( $F(5)=2.47$ ,  $p=0.06$ ) and CJ2100 ( $F(5)=2.14$ ,  $p=0.09$ ) failed to significantly impair spatial memory up to doses of 10 mg/kg.

The muscarinic agonist, arecoline, also disrupted responding on the psychomotor vigilance task. The pattern of disruption following arecoline administration was distinct from that following administration of scopolamine. Arecoline had a fast onset of effect and completely eliminated responding. After an interval of time that was related to the dose of arecoline, responding returned to control levels. The action of 3.2 mg/kg arecoline likely was through a muscarinic mechanism, since this disruption was reversed in a dose-dependent manner by scopolamine, L-687,306, and CJ2100 (Figure 7). Note that doses of each antagonist that blocked the effects of arecoline in this vigilance task were smaller than those required to affect performance in this task, indicating that the antimuscarinic properties of these drugs were present at doses below those that impaired vigilance. In addition, each of the antagonists produced complete or nearly complete reversal of the effects of 3.2 mg/kg arecoline in this task at doses that

were a log-unit smaller than those that prevented the effects of 10 mg/kg arecoline on heart rate (Fig 3).

## DISCUSSION

The discovery that the muscarinic antagonist scopolamine can rapidly and persistently reverse symptoms of depression (Drevets and Furey, 2010; Drevets et al., 2013; Drevets et al., 2020; Furey and Drevets, 2006; Witkin et al., 2020) raised the possibility that such anticholinergics might prove to be clinically useful antidepressant drugs. However, the direct adverse side-effects of short and long-term administration of scopolamine and other conventional centrally active antimuscarinic drugs and, in particular, their sedative effects and potential impact on cognitive function has limited enthusiasm for this line of medication development.

Interestingly, another rapid-onset drug with antidepressant potential, ketamine (Corrigan and Pickering 2019), also has disruptive effects on learning and memory, likely through its block of NMDA receptors that mediate long term potentiation in the hippocampus (Lüscher and Malenka, 2012). The possible interaction between NMDA and cholinergic antagonism and their potentially common influence on both mood and memory through potentiation of AMPA receptor function, has been discussed (Witkin et al., 2019). Less often raised is the question of whether cognitive impairment and memory deficits produced by the anticholinergic scopolamine and the NMDA antagonist ketamine can be meaningfully dissociated from beneficial antidepressant effects. Directly related to this, a relatively recent SSRI with mixed serotonergic activity, vortioxetine, actually improves cognitive performance in depressed patients (Frampton, 2016),

indicating that antidepressant action is not inextricably linked with memory disturbance. The present data further support the separation of cognitive impairment and antidepressant activity by demonstrating that two novel muscarinic antagonists, L-687,306 and CJ2100, at doses that are equi-effective with scopolamine in a preclinical assay of potential antidepressant effectiveness, do not impair cognitive functions in rodent models of memory and attention. Although no direct comparison of the three compounds was made on measures of sedation (e.g., sleep-wake cycles), it was found that a dose of 3 mg/kg of either L-687,306 or CJ2100 did not suppress ongoing rates of food-maintained behavior (data not shown). Scopolamine, on the other hand, completely suppressed these rates at a dose of 0.3 mg/kg (data not shown). It is also worthwhile to note that Freedman et al. (1993) stated that at doses up to 30 mg/kg, L-687,306 was unique among a group of muscarinic antagonists in that “no salivation, diarrhea, tremor, hypothermia or other side effects normally associated with muscarinic stimulation *in vivo*” (page 493) was observed.

Like scopolamine, L-687,306 and CJ2100 are effective muscarinic antagonists in that they each completely antagonized the disruptive effects of the muscarinic agonist arecoline on heart rate, an autonomically-mediated effect and on psychomotor vigilance, a centrally-mediated effect. Interestingly, the three drugs did not display the same rank order potency as antagonists in these two tasks. For example, L-687,306 and CJ2100 were equipotent in attenuating arecoline-induced bradycardia, whereas scopolamine was approximately 10 times more potent than either drug in this assay. In the psychomotor vigilance assay, scopolamine was equipotent with L-687,306 as an arecoline antagonist, and approximately 10 times more potent than CJ2100. This

difference in rank-order potency suggests that the antagonists are acting through distinct receptors or sets of receptors to block the effects of arecoline in the two assays. The bradycardia produced by arecoline on heart rate likely is mediated through the M<sub>2</sub> receptor (Harvey 2019), whereas the type of muscarinic receptors mediating the central effects of arecoline are unknown.

Each of the three antimuscarinic compounds also had similar direct effects in the FST, decreasing immobility and increasing either swimming or climbing. Importantly, scopolamine and CJ2100 decreased immobility in the FST without significantly altering locomotor activity at the same doses, suggesting that these effects in the FST were independent of general activity-stimulating properties. L687,306 significantly increased locomotor activity; however, these effects were less robust than that observed with psychomotor stimulants in the same apparatus (Altshuler et al., 2019). These data highlight that locomotor-stimulating properties do not necessarily predict behaviors in the FST. Further, the FST has been validated as an animal assay of antidepressant activity (Krishnan and Nestler, 2011; Yankelevitch-Yahav et al., 2015) and, consistent with its effects in this assay, scopolamine has been found to have a robust, fast-acting antidepressant action in some clinical studies (Drevets and Furey, 2010; Drevets et al., 2013; Furey and Drevets, 2006). The present findings raise the possibility that L-687,306 and CJ2100 also might be clinically effective antidepressants.

Despite the finding that all three antagonists could reverse the effects of arecoline in an autonomic assay, and that all three decreased immobility in the FST, scopolamine differed qualitatively from either L-687,306 or CJ2100 in drug discrimination and cognition assays. As reported previously, behaviorally active doses

of scopolamine did not consistently block the discriminative stimulus effects of arecoline. It failed to attenuate, or even potentiated the rate decreasing effects of arecoline, other mAChR agonists, and acetylcholinesterase inhibitors (Jung et al., 1987; Yamamoto et al., 1993; Winger et al., 2020). On the other hand, L-687,306 and CJ2100 produced rightward displacements in the discriminative stimulus and rate-suppressing effects of arecoline, indicative of muscarinic antagonism. Scopolamine's effects also differed from those of the other two muscarinic antagonists in vigilance and delayed matching-to-position studies. In both these measures of cognitive function, CJ2100 or L-687,306 had little effect over a wide range of doses that were beyond the effective dose in the FST assay. In contrast, the dose of scopolamine that was similarly effective in the FST assay (0.32 mg/kg) produced clear disruptions in performance of both tasks. The data revealed at least a 3-fold separation in the potency with which L-687,306 and CJ2100 produced desirable (decreased immobility in FST) and undesirable (cognitive impairment) effects. In sharp contrast, scopolamine displayed no separation between desirable and undesirable effects. With regard to the potential clinical application of novel antimuscarinics such as L-687,306 or CJ2100, e.g., in the treatment of depression, these results indicate that the projected therapeutic window may markedly improve over that of conventional antagonists like scopolamine.

Results of the present studies illustrate clear differences in the direct effects of antimuscarinics. In previous studies, Witkin et al. (1987) also noted that the behavioral effects of antimuscarinics can differ among ligands. For example, they found that schedule-controlled responding in rats was decreased by four of seven antimuscarinic compounds and initially increased by the remaining three compounds. In conjunction

with such observations, the present findings emphasize the point that differences in the behavioral profiles of antimuscarinic drugs perhaps can be exploited to identify clinically valuable therapeutics. Thus, all antimuscarinic compounds can block at least some effects of muscarinic agonists like arecoline, but not all produce the degree of cognitive impairment that is often associated with scopolamine.

The differences among the behavioral effects of antimuscarinic compounds may reflect differences in their relative affinity and efficacy at the five subtypes of muscarinic receptors. Witkin et al (2014) reported that  $M_1$  and  $M_2$  null mice were not responsive to the effects of scopolamine in a forced swim assay, while  $M_3$ ,  $M_4$ , and  $M_5$  null mice continued to respond normally to scopolamine. This implicates  $M_1$  and  $M_2$  receptor subtypes in the antidepressant actions of scopolamine. The antagonism by all three of the compounds described in this manuscript on the bradycardia produced by arecoline indicates activity at the  $M_2$  receptor. However, the lack of mAChR subtype specificity of L- 687,306 and CJ2100, as shown in the Table, does not provide any additional information on what receptor subtype might mediate the cognitive deficits produced by some anticholinergic compounds.

Studies with compounds that have greater specificity for the  $M_1$  receptor such as biperidine, pirenzepine and VU0255035 (reviewed in Witkin et al., 2020) indicate that they have antidepressant-like activity in rodent assays, supporting a role for  $M_1$  receptors in mediating the antidepressant effects of cholinergic antagonists. Likewise, SCH226206, an  $M_2$  subtype-selective compound had antidepressant-like actions in mice (Witkin et al. 2014; reviewed in Witkin et al., 2020). There is therefore consistent support for the importance of the  $M_1$  and  $M_2$  receptor subtypes in mediating

antidepressant activity. Whether the cognitive impairment produced by scopolamine is due to additional action at the other three subtypes, and why CJ2100 and L 687,306 generally lack these side effects remains to be determined.

There are a few caveats regarding the present studies that warrant consideration. First, with the exception of the cognition studies, only male rats were examined. Diagnoses of MDD are more prevalent in women (Hyde and Mezulis, 2020) and, therefore, in future preclinical efforts in muscarinic drug development with L-687,306 and CJ2100, it will be important to determine whether these drugs produce similar discriminative stimulus and FST effects in female rats (Kokras et al., 2015). Second, the forced swim test is an assay often used to rapidly screen compounds for potential antidepressant-like activity but is thought to have little face validity for modeling depression or depressive symptoms in patients. While there is much debate in the field whether any animal model can adequately model human depression, future studies should aim to evaluate the consistency of these muscarinic antagonists across the many assays used to evaluate novel antidepressant compounds. Finally, although it remains important to evaluate the binding characteristics of promising compounds, it must be recognized that unique blends of activity—either combined or cooperative (Witkin et al., 1987; Leaderbrand et al., 2016)—at more than one receptor may be necessary to produce some of the distinctive behavioral effects of muscarinic antagonists (e.g. functional selectivity). Under such circumstances, the role of *in vivo* assays that are well-validated and highly translatable to clinical conditions becomes more prominent. The assessment here of the effects of novel muscarinic antagonists on different types of cognitive function serves as an example of the value of this approach.

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## **AUTHORSHIP CONTRIBUTIONS**

Participated in research design: Johnson, Kangas, Jutkiewicz, Winger, Bergman, Coop, Woods

Conducted experiments: Johnson, Kangas, Jutkiewicz, Winger

Performed data analysis: Johnson, Kangas, Jutkiewicz, Winger

Wrote or contributed substantially to the writing of this manuscript: Johnson, Kangas, Jutkiewicz, Winger, Bergman, Coop, Woods

All authors have approved of the final version of this manuscript.

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## FOOTNOTES

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|             | K <sub>i</sub> (nM) |                |                |                |                | Off-target  |
|-------------|---------------------|----------------|----------------|----------------|----------------|---|
|             | M <sub>1</sub>      | M <sub>2</sub> | M <sub>3</sub> | M <sub>4</sub> | M <sub>5</sub> |   |
| L-687,306   | 70.13               | 77.47          | 15.31          | 44.68          | 14.57          | 5HT <sub>3</sub> : 1591.12<br>D <sub>3</sub> : 4083.48                |
| CJ2100      | 353.83              | 182.91         | 71.21          | 283.77         | 159.96         | 5HT <sub>2B</sub> : 1670.66<br>D <sub>1</sub> : >10000<br>MOR: >10000 |
| Scopolamine | 2.16                | 8.20           | 0.19           | 0.80           | 0.91           |   |

Table 1. *In vitro* binding profiles of L-687-306, CJ2100, and scopolamine. Full binding profiles were performed through the PDSP. For primary binding assays, compounds showing a minimum of 50% inhibition at 10μM are screened for secondary binding assays to determine K<sub>i</sub> values (11 concentrations in triplicate).

## LEGENDS FOR FIGURES

**Figure 1.** Synthesis of L-687,306.

**Figure 2.** Synthesis of CJ2100.

**Figure 3.** Antagonism by 0.3 mg/kg CJ 2100 (closed triangles), and 1.0 mg/kg CJ2100 (open squares), of the brief, profound bradycardia produced by s.c. administration of 10 mg/kg arecoline (closed circles). Saline control data and the effects of the smallest, most effective doses of L 687,306 and scopolamine are shown in the dashed and dotted lines for comparison with CJ 2100. Eight rats comprised the arecoline and saline data, and four rats each comprised the effects of the antagonists. Data were fitted ( $R^2=0.77$ ) with a linear mixed model using treatment and time as fixed factors and subject ( $n=12$ ) as random factor (Jamovi version 1.2). The effects of treatment ( $F[4,180]=57.80$ ), time ( $F[9,216]=20.1$ ), and the treatment x time interaction ( $F[36,216]=8.11$ ) were each statistically significant ( $p<0.001$ ). Bonferroni-corrected multiple comparisons among the cells means that the results obtained with arecoline were significantly different from saline at 2-5 min only ( $p<0.001$ ). At 2-4 min, the results obtained in all three pretreatment conditions were significantly different from arecoline ( $p<0.001$ ) and not from saline. Note that the initial bradycardia is an artifact of handling and injecting the rats and occurs too rapidly to be a drug effect.

**Figure 4. Top:** the interaction between scopolamine (A), L-687,306 (B) and CJ2100 (C) on the discriminative stimulus effects of arecoline (closed circles). **Bottom:** simultaneous measures of the interactions of these drugs on the rates at which the rats carried out the discrimination task. The effect of saline on arecoline discrimination and rates of responding are shown as open circles.  $n=6-8$ .

**Figure 5.** Effects of several doses of scopolamine (A), L-687,306 (B) and CJ2100 (C) on counts of immobility, swimming, and climbing in the forced swim test.  $n=6$  rats per dose per drug;  $*p<0.05$ ,  $**p<0.01$ ,  $***p<0.001$ . Locomotor activity was evaluated at doses that produced significant decreases in immobility in the FST (D) which demonstrate significant main effects of treatment [ $F(3,28) = 6.6, P=0.002$ ] and time [ $F(10,280)=31.6, P<0.0001$ ].

**Figure 6.** (A) Dose-related effects of scopolamine (closed squares), L-687,306 (closed diamonds) and CJ2100 (closed circles) on mean titrated duration in sec in the psychomotor vigilance task  $n=6$ . (B) Dose-related effects of the three muscarinic antagonists on the mean titrated delay in the titrating delay matching-to-position task.  $n=6$ ;  $**p<0.01$ ,  $***p<0.001$ .

**Figure 7.** Antagonism of the effects of 3.2 mg/kg arecoline on the psychomotor vigilance task by a range of doses of scopolamine (A), L-687,306 (B), and CJ2100 (C).  $n=6$

Figure 1

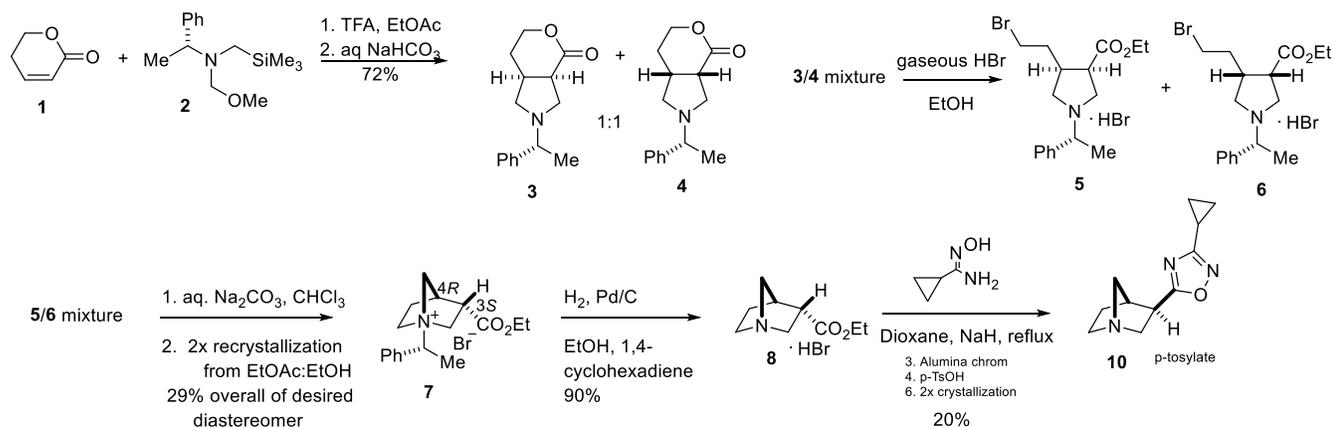


Figure 2

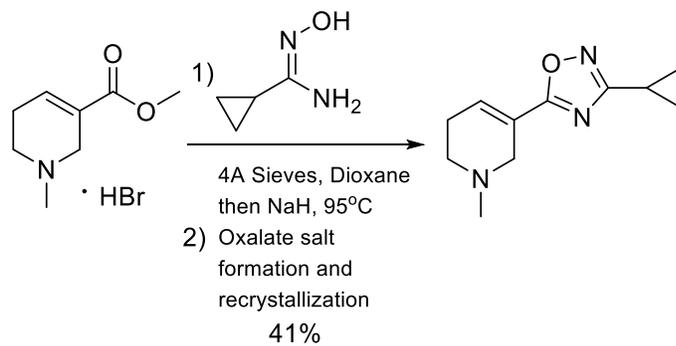


Figure 3

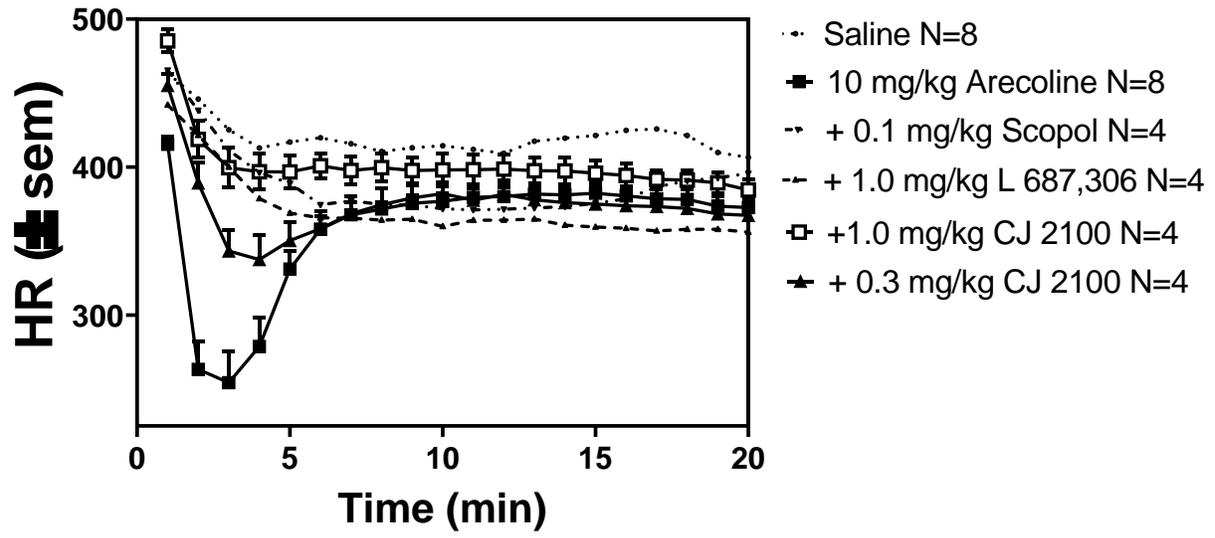


Figure 4

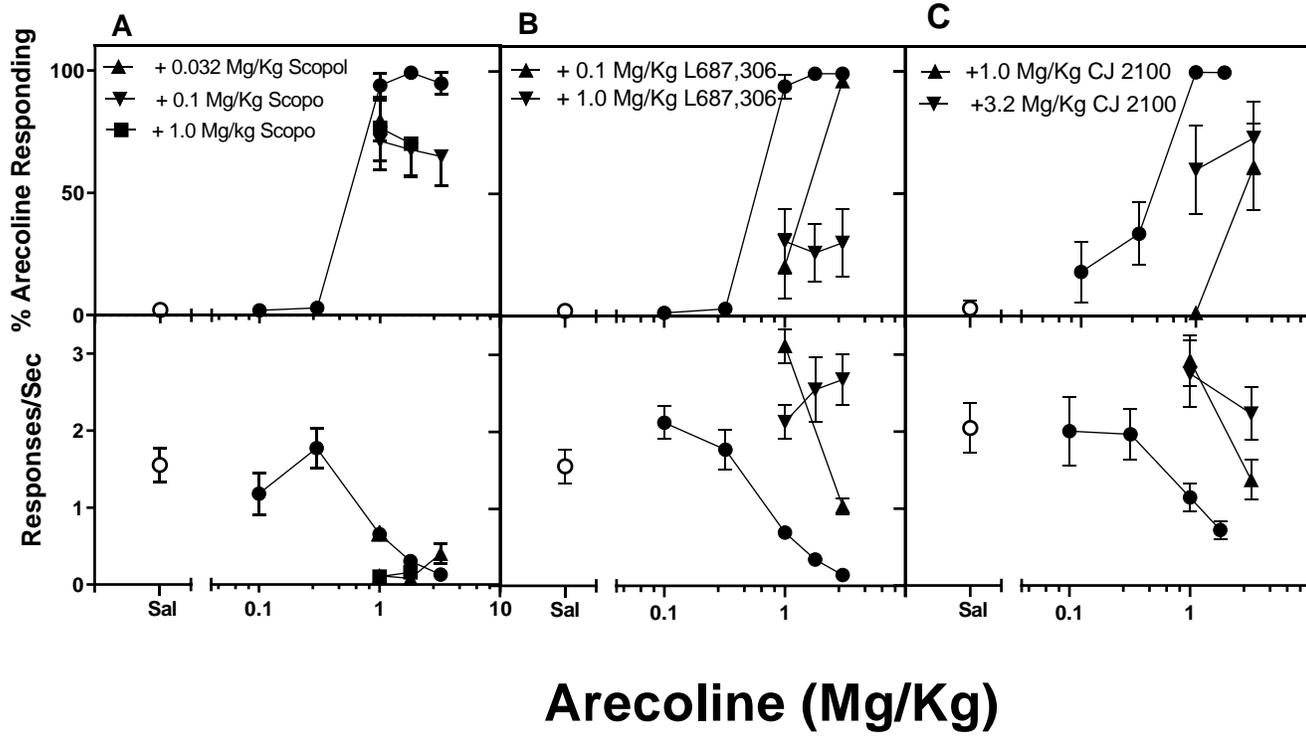


Figure 5

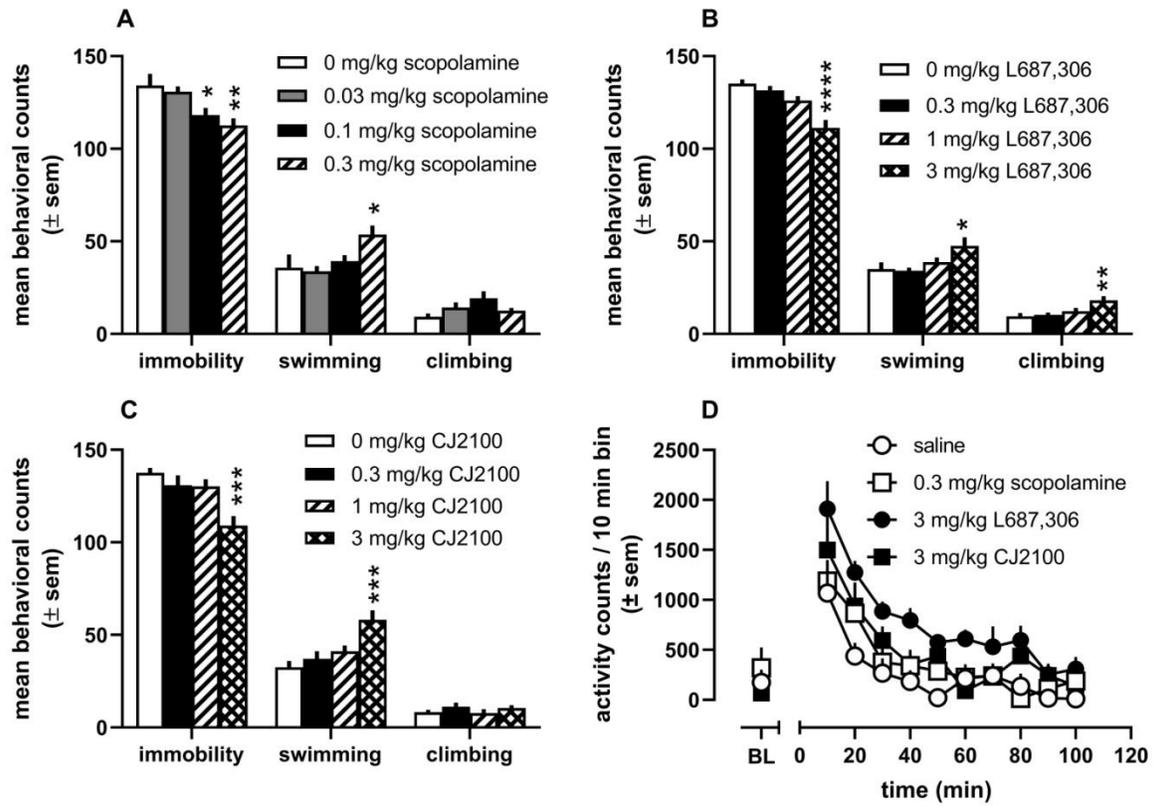


Figure 6

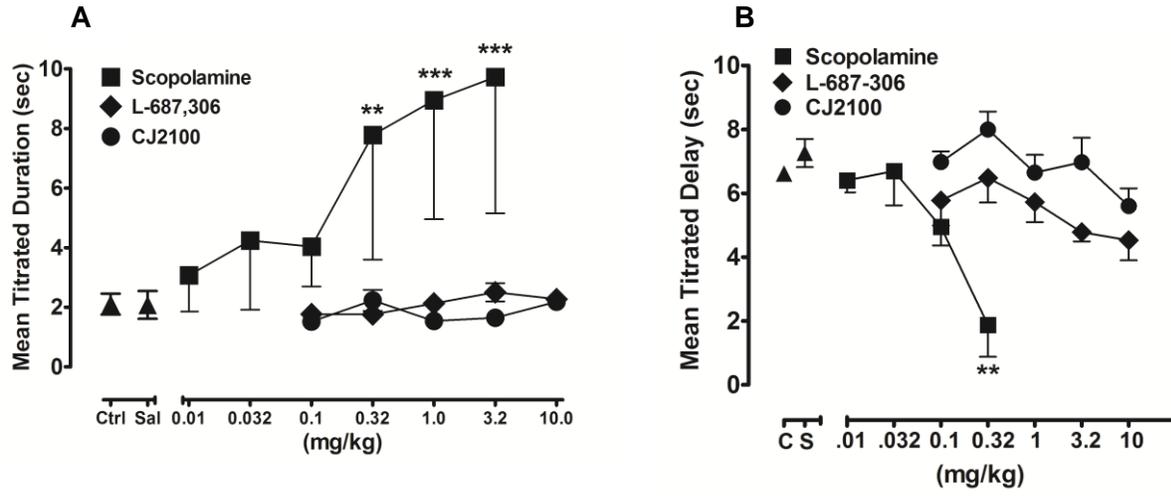


Figure 7

