

Discriminative Stimulus Properties of the Endocannabinoid Catabolic Enzyme Inhibitor SA-57 in Mice

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2-arachidonoylglycerol (2-AG); cannabinoid-1 (CB₁) receptor; cannabinoid-2 (CB₂) receptor; CP55,940 ((-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol); fatty acid amide hydrolase (FAAH); monoacylglycerol lipase (MAGL); alpha/beta-hydrolase domain 6 (ABHD6); N-arachidonoyl ethanolamine (anandamide; AEA); rimonabant (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide); PF-3845 (N-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide) JZL184 (4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester); JZL195 (4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate); MJN110 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate); SA-57 (4-[2-(4-Chlorophenyl)ethyl]-1-piperidinecarboxylic acid 2-(methylamino)-2-oxoethyl ester); SR144,528 (N-[(1S)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide); URB597 (Cyclohexylcarbamic acid 3'-(Aminocarbonyl)-[1,1'-biphenyl]-3-yl ester); KT195 ([4-(4'-Methoxy[1,1'-biphenyl]-4-yl)-1H-1,2,3-triazol-1-yl](2-phenyl-1-piperidinyl)-methanone); KT182 ([4-[3'-(Hydroxymethyl)[1,1'-biphenyl]-4-yl]-1H-1,2,3-triazol-1-yl](2-phenyl-1-piperidinyl)-methanone).

e) Recommended section: Behavioral pharmacology

Abstract

Whereas inhibition of fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL), the respective major hydrolytic enzymes of N-arachidonoyl ethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) elicits no or partial substitution for Δ^9 -tetrahydrocannabinol (THC) in drug discrimination procedures, combined inhibition of both enzymes fully substitutes for THC, as well as produces a constellation of cannabimimetic effects. The present study tested whether C57BL/6J mice would learn to discriminate the dual FAAH-MAGL inhibitor SA-57 (4-[2-(4-chlorophenyl)ethyl]-1-piperidinecarboxylic acid 2-(methylamino)-2-oxoethyl ester) from vehicle in the drug discrimination paradigm. In initial experiments, 10 mg/kg SA-57 fully substituted for CP55,940 ((-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol), a high efficacy CB₁ receptor agonist in C57BL/6J mice and for AEA in FAAH (-/-) mice. The majority (i.e., 23 of 24) of subjects achieved criteria for discriminating SA-57 (10 mg/kg) from vehicle within 40 sessions, with full generalization occurring 1-2 h post injection. CP55,940, the dual FAAH-MAGL inhibitor JZL195 (4-nitrophenyl 4-(3-phenoxybenzyl)piperazine-1-carboxylate), and the MAGL inhibitors MJN110 (2,5-dioxopyrrolidin-1-yl 4-(bis(4-chlorophenyl)methyl)piperazine-1-carboxylate) and JZL184 (4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester) fully substituted for SA-57. Although the FAAH inhibitors PF-3845 ((N-3-pyridinyl-4-[[3-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidinecarboxamide) and URB597 (cyclohexylcarbamic acid 3'-(aminocarbonyl)-[1,1'-biphenyl]-3-yl ester) did not substitute for SA-57, PF-3845 produced a two-fold leftward shift in the MJN110 substitution dose response curve. In addition, the CB₁ receptor antagonist rimonabant blocked the generalization of SA-57 as well as substitution of CP55,940, JZL195, MJN110, and JZL184. These findings suggest MAGL inhibition plays a major role in the CB₁ receptor-mediated SA-57 training dose, which is further augmented by FAAH inhibition.

Introduction

Cannabinoid CB₁ (Devane *et al.*, 1988; Matsuda *et al.*, 1990) and CB₂ receptors (Munro *et al.*, 1993) and their endogenous ligands N-arachidonoyl ethanolamine (anandamide; AEA) (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995) represent primary elements of the endocannabinoid system. This system modulates many physiological processes, including pain (Hohmann *et al.*, 2005; Kinsey *et al.*, 2010; Woodhams *et al.*, 2012; Ignatowska-Jankowska *et al.*, 2014), memory (Hampson and Deadwyler, 1999), appetite (Kirkham and Tucci, 2006), and reward (Tsou *et al.*, 1998; Marsicano and Lutz, 1999). The primary psychoactive constituent of *Cannabis*, Δ^9 -tetrahydrocannabinol (THC) (Gaoni and Mechoulam 1964) produces its psychotomimetic effects through CB₁ receptors (Huestis *et al.*, 2001), and induces dopamine release in the nucleus accumbens (Chen *et al.*, 1991), though to a substantially lower magnitude than other abused drugs. Curiously, THC produces reinforcing effects in some (Gardner *et al.*, 1988; Lepore *et al.*, 1996; Justinova *et al.*, 2003, 2005), but not all (Vlachou *et al.*, 2007; Wiebelhaus *et al.*, 2015) preclinical laboratory animal models. In contrast, THC serves as a reliable discriminative stimulus in the drug discrimination paradigm (Henriksson *et al.*, 1975; Järbe, 1989; Wiley *et al.*, 1997; Vann *et al.*, 2009), an assay that is highly predictive of drug psychoactivity in humans (Chait *et al.*, 1988; Kamien *et al.*, 1993; Lile *et al.*, 2012).

Whereas THC elicits relatively long-lasting pharmacological effects, AEA and 2-AG produce short-lived effects because of rapid hydrolysis by their respective primary catabolic enzymes fatty acid amide hydrolase (FAAH) (Cravatt *et al.*, 1996, 2001) and monoacylglycerol lipase (MAGL) (Di Marzo *et al.*, 1999; Dinh *et al.*, 2002). Accordingly, inhibitors of these enzymes elevate endocannabinoid brain levels, and represent useful investigative tools. Although the selective FAAH inhibitors URB597 (Fu *et al.*, 2005) and PF-3845 (Ahn *et al.*, 2009) elevate AEA brain levels and produce antinociceptive effects, neither compound substitutes for THC (Gobbi *et al.*, 2005; Wiley *et al.*, 2014). Similarly, the MAGL

inhibitor JZL184 elevates endogenous 2-AG brain levels and produces antinociception, but only partially substitutes for THC (Long et al. 2008; Long et al. 2009; Wiley et al. 2014; Walentiny et al. 2015). Conversely, the dual FAAH-MAGL inhibitor JZL195 fully substitutes for THC, elicits a constellation of cannabimimetic effects (Long *et al.*, 2009; Wise *et al.*, 2012; Hruby *et al.*, 2015) and produces an increased magnitude of antinociceptive effects compared with single enzyme inhibition (Long *et al.*, 2009; Ghosh *et al.*, 2015). Similarly, the dual FAAH-MAGL inhibitor SA-57 fully substitutes for THC in wild-type mice (Hruby et al. 2015).

As it has yet to be established whether an inhibitor of endocannabinoid hydrolysis can serve as the training drug in drug discrimination procedures, the present study investigated whether mice will learn to discriminate SA-57 from vehicle. SA-57 inhibits FAAH much more potently than it inhibits MAGL or ABHD6, another serine hydrolase that degrades 2-AG, but to a much less extent than MAGL (Blankman *et al.*, 2007). Thus, SA-57 possesses utility to investigate the consequences of maximally elevating brain AEA levels, while dose-dependently increasing brain 2-AG levels (Niphakis *et al.*, 2012). To select the SA-57 training dose, initial experiments examined its dose-effect relationship to substitute for the potent CB₁ receptor agonist CP55,940 in C57BL/6J mice and AEA in FAAH (-/-) mice (to prevent rapid hydrolysis). Having established that mice learn to discriminate SA-57 from vehicle, we then assessed its dose-response relationship and time course. Because various substrates of FAAH (e.g., AEA, palmitoylethanolamide (PEA), and oleoylethanolamide (OEA)) and MAGL (e.g., 2-AG) bind CB₁, CB₂, TRPV1 (Smart *et al.*, 2000), and peroxisome proliferator-activated receptor-alpha (PPAR α) receptors (Lo Verme *et al.*, 2005), we tested whether antagonists for these receptors would block the discriminative stimulus effects of SA-57. Additionally, we conducted an extensive series of drug substitution tests to gain further insight into the training dose of the SA-57 discriminative stimulus. Specifically, we tested whether CP55,940, as well as the non-cannabinoid psychoactive drugs nicotine and diazepam would substitute for the SA-57. As MAGL also plays a rate limiting role in the production

of arachidonic acid and prostanoids in brain (Nomura *et al.*, 2011), we examined whether the COX-2 inhibitor valdecoxib, which reduces prostanoid synthesis but does not affect brain endocannabinoid levels, would substitute for SA-57. The final goal of the present study was to elucidate the degree to which relevant endocannabinoid hydrolytic enzyme inhibitors contribute to the SA-57 training dose. Accordingly, we investigated whether individual FAAH, MAGL, and ABHD6 inhibitors, we well as simultaneous inhibition of FAAH and MAGL would substitute for SA-57.

Materials and methods

Subjects

Male C57BL6/J mice (Jackson Laboratory; Bar Harbor, ME) and male FAAH (-/-) mice served as subjects. The FAAH (-/-) mice were backcrossed >14 generations on to a C57BL6/J background. The mice were 9-11 weeks of age at the beginning of training and were individually housed in a temperature-controlled (20-22°C) vivarium in accordance with Virginia Commonwealth University Institutional Animal Care and Use Committee guidelines. Mice were given water ad libitum, and were food restricted to 85-90% of free-feed body weight, which was established during a two-week period of ad libitum food every six months.

Drugs

SA-57, MJN110, KT182, KT195, and JZL195 were synthesized in the Cravatt laboratory, as previously described (Long *et al.*, 2009; Niphakis *et al.*, 2012, 2013; Hsu *et al.*, 2013). N-arachidonoyl ethanolamine (AEA) was provided by Organix Inc. (Woburn, MA), and valdecoxib was provided by Sigma-Aldrich (Saint Louis, MO). CP55,940, JZL184, PF-3845, rimonabant, and SR144528 were generously supplied by the National Institute on Drug Abuse (NIDA) (Rockville, Maryland, USA). Capsazepine was purchased from Cayman Chemical, and GW6471 was purchased from Tocris Bioscience. Each compound was dissolved in a vehicle consisting of ethanol, emulphor-620 (Rhodia, Cranbury, New Jersey, USA), and saline in a ratio of 1:1:18. All injections were given via the intraperitoneal (i.p.) route of administration in a volume of 10 µl per 1 g of body weight.

Apparatus

Drug discrimination was conducted in eight sound-attenuating operant conditioning boxes (18 x

18 x 18 cm) (MED Associates, St. Albans, VT). Each operant box contained two nose poke apertures, and a food dispenser delivering 14-mg food pellets to a receptacle chamber located between apertures. Computer software (MED-PC[®] IV, MED Associates, St. Albans, VT) was used to record nose pokes and to control stimulus presentations and food deliveries.

Drug Discrimination Paradigm

Training

Separate groups of mice were trained to discriminate each of the following three training drugs from vehicle. Groups 1 and 2 consisted of C57BL6/J mice (n=8) trained to discriminate CP55,940, and FAAH (-/-) mice (n=11) trained to discriminate AEA, respectively. The third group of mice consisted of three cohorts of C57BL6/J mice (n=8/cohort) trained to discriminate SA-57 from vehicle. The treatment conditions for each cohort are described below under Testing. The pretreatment times for the training drugs were 120 min for SA-57 and 30 min for CP55,940 and AEA. During each 15 min training session, both nose poke apertures were available, but only responses into the correct aperture associated with the appropriate training drug or vehicle resulted in food reinforcement. Each incorrect response reset the response requirement. Injections before training sessions were conducted (Monday-Friday) in a double alternation sequence of drug (SA-57, CP55,940, or AEA) and vehicle (e.g., vehicle, vehicle, drug, drug).

Testing

Test sessions were scheduled twice per week, with a minimum of 72 h between test days. To be eligible for testing, subjects were required to meet the following three criteria on nine of the previous ten consecutive training sessions: 1) correct completion of the first FR10 (i.e., first 10 consecutive responses into the appropriate aperture); 2) $\geq 80\%$ correct responding; and 3) maintain response rates ≥ 10 responses/min. During the 15-min test sessions, responses in either aperture resulted in the delivery of

food reinforcement according to an FR10 schedule of reinforcement, without a limitation on the number of reinforcers earned within a session. Before conducting substitution tests, dose-response tests with SA-57, CP55,940 or AEA were conducted to characterize their generalization gradients to their respective discriminative stimulus. For time course studies, animals were injected with SA-57 (10 mg/kg) and tested at 0.25, 1, 2, 4, or 8 h after injection. In order to assess whether CB₁ receptors mediated the discriminative effects of SA-57, and the substitution of CP55,940, MJN110, JZL184, and JZL195, we used rimonabant (3 mg/kg; Rinaldi-Carmona, 1994). We also examined whether the CB₂ receptor antagonist SR144528 (3 mg/kg; Rinaldi-Carmona et al., 1998), the TRPV1 receptor antagonist capsazepine (5 mg/kg; Kinsey et al. 2009), and the PPAR α receptor antagonist GW6471 (2 mg/kg; Lo Verme et al. 2005) would block the discriminative stimulus effects of SA-57. Each antagonist was administered 15 min prior to injections of 10 mg/kg SA-57. The three cohorts of mice trained to discriminate SA-57 were employed in the following experiments. All cohorts were included in the SA-57 acquisition curve. Cohort 1 was used in the time-course study, the MJN110 (0.25 – 5 mg/kg), KT182 (1 and 2 mg/kg), KT195 (40 mg/kg), valdecoxib (10 mg/kg), and MJN110 (2.5 mg/kg) + PF3845 (10 mg/kg) substitution studies; cohort 2 was used to test the psychoactive non-cannabinoid drugs nicotine (1.5 mg/kg) and diazepam (10 mg/kg), and in substitution tests with JZL195 (2-20 mg/kg), JZL184 (4-100 mg/kg), PF3845 (10 and 30 mg/kg), and URB597 (10 mg/kg); and cohort 3 was used in the receptor antagonist experiments (rimonabant, SR144528, capsazepine, GW6471).

[³H]SR141716A binding assay

Cerebella were dissected from adult male ICR mice, stored at -80°C, and membranes were prepared as described previously (Selley *et al.*, 2004). Membrane protein (15 μ g) was incubated with 0.94 nM [³H]SR141716A in assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl₂ and 0.2 mM EGTA) with 0.5% (wt/vol) bovine serum albumin (BSA) in the presence and absence of 5 μ M unlabeled

SR141716A to determine non-specific and specific binding, respectively. The assay was incubated for 90 min at 30°C and terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters that were pre-soaked in Tris buffer containing 0.5% (wt/vol) BSA (Tris-BSA), followed by five washes with cold Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency in ScintiSafe Econo 1 scintillation fluid after a 12-h delay.

Data analysis

The percentage of drug appropriate responses and response rates (responses/min) were recorded for each experiment. Full substitution was defined as greater than or equal to 80% nose pokes that occurred into aperture associated with the training drug. Partial substitution was defined as greater than or equal to 20% and less than 80% nose pokes in the training drug-paired aperture. Less than 20% nose pokes on the drug-paired aperture was defined as no substitution (Solinas *et al.*, 2006). ED₅₀ values (and 95% confidence intervals) for generalization or substitution were calculated using least squares linear regression analysis. Behavioral data are depicted as mean ± S.E.M. The data were analyzed using one-way or two-way ANOVA. Dunnett's tests or Bonferroni post hoc analyses were used following a significant ANOVA for the response rate data. GraphPad Prism 6.0 statistical software (Graph Pad Software, Inc., La Jolla, CA) was used for data analysis.

Binding data were determined in triplicate and are reported as specific binding. Each competition dataset was analyzed by one-way ANOVA to determine concentration-dependence. Rimonabant competition curves were analyzed by non-linear regression to determine IC₅₀ and Hill coefficients using a four parameter fit with GraphPad Prism 6.0. The IC₅₀ values were then converted to K_i values using the Cheng-Prusoff equation.

Results

SA-57 substitutes for CP55,940 in C57BL/6J mice and AEA in FAAH (-/-) mice

Figure 1 shows that SA-57 fully substituted for CP55,940 and AEA in mice trained to discriminate each of these drugs. C57BL/6J mice administered either CP55,940 or SA-57 completely occasioned the discriminative stimulus effects of CP55,940 (Figure 1A). SA-57 did not affect response rates; however, CP55,940 significantly reduced response rates [$F(4,55) = 4.7$; $p < 0.01$], with 0.2 mg/kg yielding significant reductions in response rates compared with vehicle (Figure 1B). In FAAH (-/-) mice trained to discriminate AEA (6 mg/kg) from vehicle, SA-57 also fully substituted for AEA (Figure 1C). FAAH (-/-) mice administered AEA (1-30 mg/kg) or SA-57 (1-10 mg/kg) dose-dependently selected the aperture associated with AEA (Figure 1C). Both AEA [$F(4, 50) = 27.5$; $p < 0.001$] and SA-57 [$F(5, 46) = 15.27$; $p < 0.001$] significantly reduced response rates (Figure 1D). The highest doses tested of AEA (i.e., 30 mg/kg) and SA-57 (i.e., 17 mg/kg) significantly depressed response rates compared with vehicle in FAAH (-/-) mice.

The SA-57 discriminative stimulus

Because 10 mg/kg SA-57 fully substituted for CP55,940 in C57BL/6J mice and for FAAH (-/-) mice, this dose of SA-57 was selected as the training dose in three naïve cohorts of mice ($n = 8$ mice/group). As shown in Figure 2, 50% of mice achieved the criteria to discriminate SA-57 from vehicle by the 27th training session, and 23 of 24 mice acquired the discrimination by day 40. The final mouse achieved criteria on day 74 of training, but was excluded from subsequent experiments because of its substantial delay in acquisition. Similar rates of acquisition were found for CP55,940 in C57BL/6J mice and AEA in FAAH (-/-) mice.

Figure 3 shows the time course effects of 10 mg/kg SA-57 vs. vehicle for selecting the aperture associated with SA-57 (Figure 3A) and response rates (Figure 3B). Whereas mice that received vehicle

responded consistently on the vehicle-associated aperture at each of the time points, mice administered 10 mg/kg SA-57 selected the SA-57 aperture $\geq 80\%$ at 1 and 2 h post injection, showed partial substitution at 0.25 and 4 h, and responded predominantly on the vehicle aperture 8 h after injection. There were no differences in rates of responding between mice injected with vehicle or SA-57 at any time point (Figure 3B; $p = 0.48$).

As shown in Figure 4, the CB₁ receptor antagonist rimonabant (0.03-3 mg/kg), significantly blocked the SA-57 training dose. In contrast, the CB₂ receptor antagonist SR144528 (3 mg/kg), the TRPV1 receptor antagonist capsazepine (5 mg/kg), and the PPAR α receptor antagonist GW6471 (2 mg/kg) did not block the SA-57 training dose (Table 1).

SA-57 does not bind CB₁ receptors

As the SA-57 discriminative stimulus required CB₁ receptor activation, we next examined whether this compound interacts directly with CB₁ receptors. Accordingly, we tested if SA-57 would displace [³H]SR141716A binding in mouse cerebellar membranes. As shown in Figure 5, rimonabant (i.e., unlabeled SR141716A) inhibited [³H]SR141716A binding in a concentration-dependent manner ($p < 0.001$, $F = 17.36$, $df = 7$), with a K_i value of 0.75 ± 0.16 nM and Hill coefficient of 0.97 ± 0.08 . In contrast, SA-57 (0.01 to 10 μ M) did not inhibit [³H]SR141716A binding ($p = 0.96$; Figure 5), indicating that this compound does not directly interact with CB₁ receptors.

Substitution tests in SA-57 discriminating mice

We next tested whether the non-cannabinoid, psychoactive compounds, nicotine and diazepam, would substitute for SA-57. As shown in Figure 6A, nicotine did not substitute for SA-57, but diazepam produced partial substitution. Both drugs significantly reduced response rates [Figure 6B; $F(3,28) = 14.01$; $p < 0.001$], demonstrating that behaviorally active doses were reached.

Figure 7 shows the dose-effect curves of the mixed CB₁/CB₂ receptor agonist CP55,940, the dual FAAH-MAGL inhibitor JZL195, and SA-57 in mice trained to discriminate SA-57 (10 mg/kg) from vehicle. CP55,940, JZL195, and SA-57 produced dose-related responding into the aperture associated with SA-57 (Figure 7A). CP55,940 [F (3,28) = 2.99, *p* < 0.05] and SA-57 [F (4,42) = 2.78, *p* < 0.05], but not JZL195, reduced response rates (Figure 7B).

SA-57 generalized to itself in a dose-dependent fashion, and the MAGL inhibitors MJN110 and JZL184 dose-dependently substituted for SA-57 (Figure 8A). Although MJN110 did not affect response rates [F (6, 48) = 0.33, *p* = 0.92], the highest doses of SA-57 (17 mg/kg) [F (5, 42) = 3.391, *p* < 0.05] and JZL184 (100 mg/kg) [F (4, 18) = 3.985, *p* < 0.05] significantly reduced response rates (Figure 8B). As shown in Figure 9A, rimonabant (3 mg/kg) completely blocked substitution of MJN110 (5 mg/kg), JZL184 (100 mg/kg), and JZL195 (20 mg/kg) for the SA-57 training dose. Also, rimonabant significantly reduced rates of responding [Figure 9B; F (1, 29) = 11.91, *p* < 0.01].

In contrast, mice administered high doses of the FAAH inhibitors PF-3845 (10 and 30 mg/kg) and URB597 (10 mg/kg) selected the vehicle aperture (Table 2). Likewise, mice given high doses of the ABHD6 inhibitors KT182 (1 and 2 mg/kg) or KT195 (40 mg/kg), as well as mice given high dose of the selective COX2 inhibitor valdecoxib (10 mg/kg) selected the vehicle aperture.

Because MAGL inhibitors, but not FAAH inhibitors, substituted for SA-57, we next examined whether full FAAH inhibition would elicit a leftward shift in the MAGL substitution dose-response curve. Accordingly, we tested the dose-response relationship of MJN110 with PF3845 (10 mg/kg) or vehicle for substitution in mice trained to discriminate SA-57 from vehicle. As shown in Figure 10A, PF-3845 elicited a significant leftward shift in the MJN110 substitution dose-response curve [potency ratio (95% CL) = 1.84 (1.3 – 2.8)]. No significant changes were found for response rates (Figure 10B).

Discussion

The present study demonstrates that mice readily learn to discriminate the dual FAAH-MAGL inhibitor SA-57 from vehicle. Specifically, the majority (i.e., 23 of 24) of subjects learned to discriminate the dual FAAH-MAGL inhibitor SA-57 from vehicle within 40 training sessions. The 10 mg/kg SA-57 training dose was previously demonstrated to produce significant increases in brain levels of AEA and 2-AG (Wiebelhaus *et al.*, 2015). As SA-57 fully blocks FAAH activity at lower doses (0.05-1 mg/kg) than those required to inhibit MAGL (1.25-12.5 mg/kg) (Niphakis *et al.*, 2012) it provided a useful tool to examine the consequences of full FAAH inhibition while incrementally elevating brain 2-AG. The observation that 1 mg/kg SA-57, which produces maximal increases in endogenous AEA without detectable increases in 2-AG (Niphakis *et al.*, 2012), did not generalize to the training dose (10 mg/kg SA-57) indicates that FAAH inhibition alone is not sufficient to occasion to the SA-57 training dose. Similarly, neither FAAH inhibitor (i.e., PF-3845 or URB597) substituted for SA-57. In contrast, the dual FAAH-MAGL inhibitor JZL195, and two MAGL inhibitors, MJN110 and JZL184, fully substituted for the SA-57 training dose, suggesting that MAGL inhibition alone may be sufficient for generalization to the 10 mg/kg SA-57 training dose. Interestingly, PF-3845 produced an approximately two-fold leftward shift in the MJN110 substitution dose-response curve. The observation that rimonabant completely blocked the discriminative stimulus effects of SA-57 indicates that CB₁ receptors play a necessary role in the subjective effects of SA-57. Similarly, rimonabant completely blocked the substitution of both MAGL inhibitors (MJN110 and JZL184) and the dual FAAH-MAGL inhibitor JZL195. These findings suggest that elevating endocannabinoid brain levels through the simultaneous blockade of FAAH and MAGL produces a CB₁ receptor mediated interoceptive stimulus.

Consistent with previous studies reporting that SA-57 or the dual FAAH-MAGL inhibitor JZL195 substitute for the THC discriminative stimulus (Long *et al.*, 2009; Hrubá *et al.*, 2015; Walentiny *et al.*, 2015), we found that SA-57 (10 mg/kg) fully substituted for the discriminative stimulus effects of

the potent cannabinoid receptor agonist CP55,940 in C57BL/6J mice and the endogenous cannabinoid AEA (6 mg/kg) in FAAH (-/-) mice. The potency of SA-57 in producing a discriminative stimulus was similar to its potency in substituting for either CP55,940 or AEA. Furthermore, SA-57's discriminative stimulus effects occurred at a training dose known to produce maximal increases in AEA and 2-AG (Niphakis *et al.*, 2012). In addition, CP55,940 fully substituted for SA-57, an effect that was completely blocked by rimonabant, further implicating a pivotal role of CB₁ receptors in these effects. Similarly, the dual FAAH-MAGL inhibitor JZL195 dose-dependently substituted for SA-57. Time-course investigation revealed that SA-57 partially generalized at 0.5 h, fully generalized at 1 and 2 h, partially generalized at 4 h, and by 8 h mice responded mostly on the aperture paired with vehicle.

It is noteworthy that MJN110 and JZL184 fully substituted for the discriminative stimulus effects of SA-57, while mice treated with a low dose of SA-57 (which inhibits FAAH and not MAGL), URB597, or PF-3845 selected the vehicle aperture. These findings suggest that MAGL inhibition represents a driving force underlying the SA-57 training dose. However, the observation that PF-3845 increased the potency of MJN110 to substitute for SA-57 suggests that FAAH inhibition increases the effectiveness of the discriminative stimulus produced by MAGL inhibition alone. The fact that 2-AG levels are approximately three orders of magnitude higher than AEA levels in wild type mouse brain (Ahn *et al.*, 2009; Long *et al.*, 2009) is consistent with the notion that MAGL inhibition elicits more prominent pharmacological effects than those produced by FAAH inhibition. Moreover, as FAAH is expressed on the post-synaptic terminal (Gulyas *et al.*, 2004), and MAGL (Dinh *et al.*, 2002) is expressed on the pre-synaptic terminal, it is plausible that AEA and 2-AG activate distinct CB₁ receptor mediated neuronal circuits.

Because AEA and 2-AG bind CB₁ and CB₂ receptors, AEA also binds TRPV1 receptors, and other FAAH substrates (i.e., PEA and OEA) bind PPAR α receptors (Lo Verme *et al.*, 2005), we examined whether selective antagonists for each of these receptors would block the discriminative

stimulus effects of SA-57. Rimonabant, but not the other receptor antagonists, completely blocked the discriminative stimulus effects of SA-57. These findings indicate that CB₁ receptor activation is required for the subjective effects of SA-57, while CB₂, TRPV1, and PPAR α receptors are dispensable. Moreover, the fact that SA-57 did not affect ligand binding to CB₁ receptors in either a competitive or non-competitive manner is consistent with the hypothesis that it increases brain endocannabinoid levels that then elicit a CB₁ receptor-mediated discriminative stimulus.

The present study also assessed whether a variety of psychoactive non-cannabinoid drugs would substitute for SA-57. Specifically, nicotine did not substitute for the SA-57 training dose, although it significantly reduced response rates. In contrast, diazepam partially substituted for SA-57, but did so at a dose that reduced response rates. Similarly, diazepam partially substitutes for THC at high doses that produce motor impairment in the rat drug discrimination paradigm (Wiley and Martin, 1999). Taken together, these studies suggest the possibility of a potential GABAergic component for CB₁ receptor-mediated discriminative stimuli. In addition, because MAGL inhibition reduces brain levels of arachidonic acid as well as various prostanoids (Nomura *et al.*, 2011), we tested whether the COX2 inhibitor valdecoxib would substitute for SA-57. However, valdecoxib was devoid of action in this assay, suggesting that prostaglandins do not play a necessary role in the discriminative effects of SA-57.

It is noteworthy that combined inhibition of FAAH and MAGL attenuates somatic signs of opioid withdrawal (Ramesh *et al.*, 2011). However, simultaneous blockade of these enzymes also elicits other cannabimimetic effects, as assessed in the tetrad assay including hypomotility, antinociception, catalepsy, and hypothermia (Long *et al.*, 2009; Anderson *et al.*, 2014; Ghosh *et al.*, 2015), as well as impaired performance in a Morris water maze spatial memory task (Wise *et al.*, 2012). These effects of dual FAAH and MAGL inhibition are similar to those of THC, whereas single inhibition of either enzyme produces a decreased spectrum and magnitude of cannabimimetic effects. Specifically, the MAGL inhibitor JZL184 produces antinociception, hypomotility, and dysregulation of thermoregulation

when challenged with manipulations that elicit hypothermia (Nass *et al.*, 2015), while FAAH inhibition produces antinociception, but not other cannabimimetic effects (Long *et al.*, 2009). However, drug discrimination is more sensitive in detecting cannabimimetic effects compared with the tetrad assay. For example, THC is more potent in producing its discriminative stimulus effects than in eliciting the full set of tetrad effects (Long *et al.*, 2009; Marshall *et al.*, 2014). Given that dual blockade of FAAH and MAGL significantly reduces locomotor activity (Long *et al.*, 2009) and SA-57 (10 mg/kg) completely inhibits FAAH and MAGL activity (Niphakis *et al.*, 2012), the lack of rate suppressive effects of the training dose of SA-57 is interesting. Similarly, THC (5.6 mg/kg) reduces locomotor activity, but does not reduce response rates in a drug discrimination procedure (Wiley *et al.*, 2005). This lack of apparent motor depression is consistent with the idea that rate suppressive effects of drugs undergo tolerance throughout the course of drug discrimination training (Solinas *et al.*, 2006).

The results of the present study suggest that SA-57 serves as a discriminative stimulus at doses that produce increased levels of both AEA and 2-AG through a CB₁ receptor mechanism of action, though elevated levels of 2-AG may be the main driving force for the SA-57 training dose. Although the brain regions mediating the discriminative stimulus effects of SA-57 and cannabinoid receptor agonists are unknown, it is noteworthy that endogenous cannabinoids, and their receptors are located in neural pathways mediating the reinforcing effects of drugs of abuse (i.e. mesolimbic dopamine pathway) (Oleson and Cheer, 2012).

In conclusion, the present study demonstrates that the dual FAAH-MAGL inhibitor SA-57 serves as a reliable discriminative stimulus. The observations that rimonabant completely blocks the SA-57 training dose, and mice trained to discriminate SA-57, CP55,940, and AEA show symmetrical substitution, strongly implicate the importance of the CB₁ receptor in this novel interoceptive stimulus. Collectively, these findings raise the provocative possibility that FAAH and MAGL serve as dual brakes

to prevent the psychoactive consequences of CB₁ receptor over-stimulation caused by elevated levels of AEA and 2-AG.

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Footnotes

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Figure legends

Figure 1. Effects of CP55,940, AEA and SA-57 on percentage of responses in training drug-paired apertures and response rates in C57BL/6J mice trained to discriminate CP55,940 (0.1 mg/kg; Panels A and B) or FAAH (-/-) mice trained to discriminate AEA (6 mg/kg; Panels C and D). A) Dose-dependent generalization of CP55,940 and dose-dependent substitution of SA-57 for the CP55,940 discriminative stimulus. The respective ED₅₀ (95% CI) values for CP55,940 generalization and SA-57 substitution in C57BL/6J mice were 0.04 (0.03 - 0.05) mg/kg and 2.4 (1.6 – 3.6) mg/kg. B) CP55,940 (0.2 mg/kg), but not SA-57, significantly decreased rates of responding compared to vehicle. C) Dose-dependent generalization of AEA and dose-dependent substitution of SA-57. The respective ED₅₀ (95% CI) values for AEA and SA-57 in FAAH (-/-) mice were 2.7 (2.3-3.1) mg/kg and 3.1 (2.8-3.4) mg/kg. D) SA-57 (17 mg/kg) and AEA (30 mg/kg) decreased rates of responding. Values represent mean ± SEM. Filled symbols indicate significant difference ($p < 0.001$) vs. vehicle; $n = 7-10$ mice/group.

Figure 2. Acquisition rates of SA-57 (10 mg/kg) in C57BL/6J mice, AEA (10 mg/kg) in FAAH (-/-) mice, and CP55,940 (0.1 mg/kg) in C57BL/6J mice trained in drug discrimination. Values represent percentage of mice that achieved criteria (see text) across days. $n = 24$ mice for SA57, 12 for AEA, and 12 for CP55,940.

Figure 3. Time course effects for occasioning the 10 mg/kg SA-57 training dose. A) Percentage of responses in the SA-57-associated aperture 0.25, 1, 2, 4, or 8 h following an injection of vehicle or SA-57 (10 mg/kg). B) SA-57 did not affect response rates at any time point after administration. Values represent mean ± SEM; $n = 7$ mice/group.

Figure 4. CB₁ receptors play a necessary role in the SA-57 discriminative stimulus. A) Rimobant (0.3-3 mg/kg) significantly attenuated the SA-57 training dose. B) Rimobant doses (i.e., 0.1, 0.3, 1, 3 mg/kg) that blocked the SA-57 training dose did not reduce response rates. Values represent mean ± SEM; n = 3-6 mice/group.

Figure 5. SA-57 does not compete with [³H]SR141716A binding to CB₁ receptors in mouse cerebellum. Data represent mean [³H]SR141716A bound (pmol/mg) ± SEM in the presence of varying concentrations of rimobant or SA-57 (n = 3). Specific binding of [³H]SR141716A in the absence of competing ligand was 1.65 ± 0.26 pmol/mg. Similar results were obtained with [³H]CP55,940 binding in membranes prepared from Chinese hamster ovary cells stably expressing the mouse CB₁ receptor, in which concentrations of up to 10 μM SA-57 did not affect binding (data not shown).

Figure 6. Substitution experiments of non-cannabinoid psychoactive drugs nicotine (1.5 mg/kg) and diazepam (10 mg/kg) for the SA-57 training dose. A) Nicotine did not substitute, while diazepam partially substituted for the SA-57 training dose. B) Nicotine (1.5 mg/kg) and diazepam (10 mg/kg) significantly reduced rates of responding. Values represent mean ± SEM. Asterisks indicate significant difference (p < 0.05) vs. vehicle; n = 7-8 mice/group.

Figure 7. Evaluation of the dose-response relationships of SA-57, CP55,940, and JZL195 to occasion the SA-57 (10 mg/kg) discriminative stimulus. A) SA-57 produced dose-dependent generalization, and CP55,940 and JZL195 dose-dependently substituted for SA-57. The respective ED₅₀ (95% CI) values for CP55,940 substitution, JZL195, and SA-57 generalization were 0.096 (0.076 – 0.121) mg/kg, 6.2 (3.5 – 10.9) mg/kg, and 4.4 (3.5 - 5.4) mg/kg. B) High doses of CP55,940 (0.2 mg/kg) or SA-57 (17 mg/kg) significantly reduced response rates. Values represent mean ± SEM. Filled symbols indicate significant

difference ($p < 0.05$) vs. vehicle; $n = 7-8$ mice/group.

Figure 8. Evaluation of the dose-response relationships of SA-57, MJN110 and JZL184 to occasion the SA-57 (10 mg/kg) stimulus. A) Dose-dependent generalization of SA-57 and dose-dependent substitution of MJN110 and JZL184. The respective ED_{50} (95% CI) values for MJN110 and JZL184 generalization and SA-57 substitution in C57BL/6J mice were 0.77 (0.53 – 1.1) mg/kg and 20.44 (11 – 37.97) mg/kg, and 4.39 (3.53 – 5.45) mg/kg. B) SA-57 (17 mg/kg) and JZL184 (100 mg/kg), significantly decreased rates of responding. Values represent mean \pm SEM. ** indicate significant difference ($p < 0.001$) vs. vehicle; $n = 7-8$ mice/group.

Figure 9. Substitution of MJN110 (5 mg/kg), JZL184 (100 mg/kg) and JZL195 (20 mg/kg) for SA-57 (10 mg/kg) requires CB_1 receptors. A) Rimonabant (3 mg/kg) completely blocked MJN110, JZL184 and JZL195 substitution. B) Rimonabant did affect response rates. Values represent mean \pm SEM; $n = 7-8$ mice/group.

Figure 10. The FAAH inhibitor PF-3845 augments the MJN110 substitution dose-response curve for SA-57 (10 mg/kg). A) PF-3845 (10 mg/kg) produced a leftward shift of the MJN110 substitution dose-response curve. B) None of the drug combinations significantly decreased rates of responding. Values represent mean \pm SEM; $n = 7-8$ mice/group.

Table 1. CB₁ receptors mediate the discriminative stimulus effects of the SA-57 (10 mg/kg) training dose. The CB₁ receptor antagonist rimonabant (3 mg/kg) significantly blocked the discriminative stimulus effects of SA-57 (10 mg/kg) as well as substitution of CP55,940 (0.1 mg/kg). The CB₂ receptor antagonist SR144528 (3 mg/kg), the TRPV1 receptor antagonist capsazepine (5 mg/kg), and the PPAR α receptor antagonist GW6471 (2 mg/kg) did not block the SA-57 (10 mg/kg) discriminative stimulus. The vehicle-vehicle and rimonabant-vehicle conditions are the same as those used in Figure 9. Values represent mean \pm SEM. n = 6-8 mice/group.

Drug	Antagonist	% SA-57 Substitution +/- SEM	Nose Pokes/min +/- SEM
Vehicle	Vehicle	12.8 \pm 9.4	38.9 \pm 3
	Rimonabant	4.0 \pm 1.2	24.9 \pm 3
	SR144528	0.7 \pm 0.3	36.6 \pm 3.8
	Capsazepine	1.3 \pm 0.4	20.1 \pm 2.6
	GW6471	0.3 \pm 2.6	24.6 \pm 3.1
SA-57	Vehicle	95.7 \pm 1.7	27.3 \pm 1.9
	Rimonabant	3.4 \pm 1.2	20.1 \pm 2.5
	SR144528	98 \pm 1.5	30.5 \pm 5.4
	Capsazepine	86 \pm 12.2	15.7 \pm 2.9
	GW6471	96.5 \pm 1.3	19.0 \pm 2
CP55,940	Vehicle	82.5 \pm 11	33.1 \pm 3.3
	Rimonabant	10.4 \pm 5.7	20.7 \pm 4.9

Table 2. FAAH inhibitors (PF-3845 and URB597), ABHD6 inhibitors (KT182 and KT195), and the COX2 selective inhibitor valdecoxib do not substitute for the discriminative stimulus effects of SA-57 (10 mg/kg) in C57BL/6J mice and do not affect response rates. Values represent mean \pm SEM; n = 7-8 mice/group.

Enzyme	Drug (mg/kg)	% SA-57 Substitution +/- SEM	(Nose Pokes/min) +/-SEM
FAAH	Vehicle	0.6 \pm 0.5	47.5 \pm 4.9
	PF-3845 (10)	1.5 \pm 0.5	34.4 \pm 3.8
	PF-3845 (30)	0.7 \pm 0.2	38.9 \pm 3.5
	URB597 (10)	2.1 \pm 1.0	36.9 \pm 5.6
ABHD6	Vehicle	1.1 \pm 0.6	46.5 \pm 2.6
	KT182 (1)	1.4 \pm 0.7	41.1 \pm 3.5
	KT182 (2)	1.4 \pm 0.8	43.5 \pm 2.4
	KT195 (40)	0.8 \pm 0.3	38.1 \pm 3.1
COX2	Vehicle	1.1 \pm 0.6	46.5 \pm 2.6
	Valdecoxib (10)	1.1 \pm 0.7	31.9 \pm 3.9

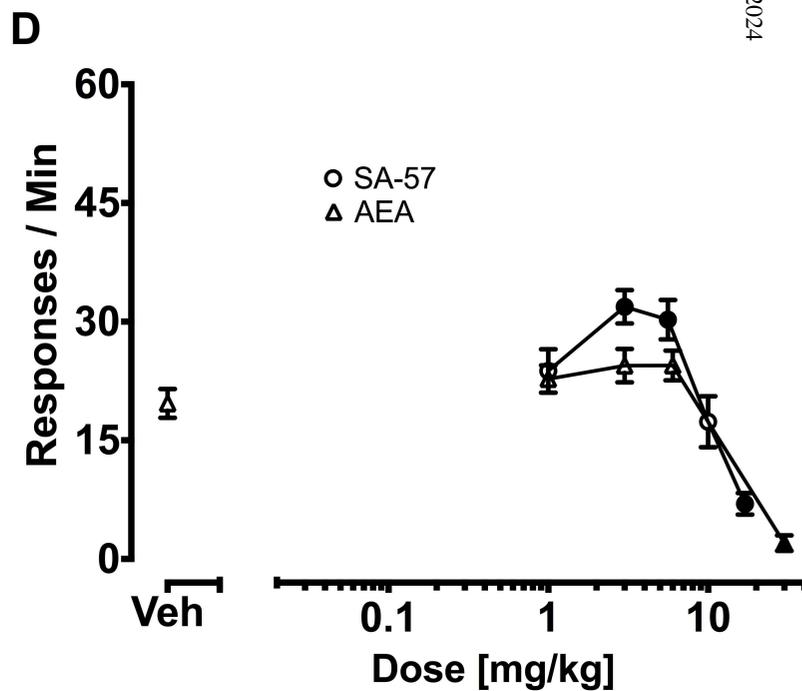
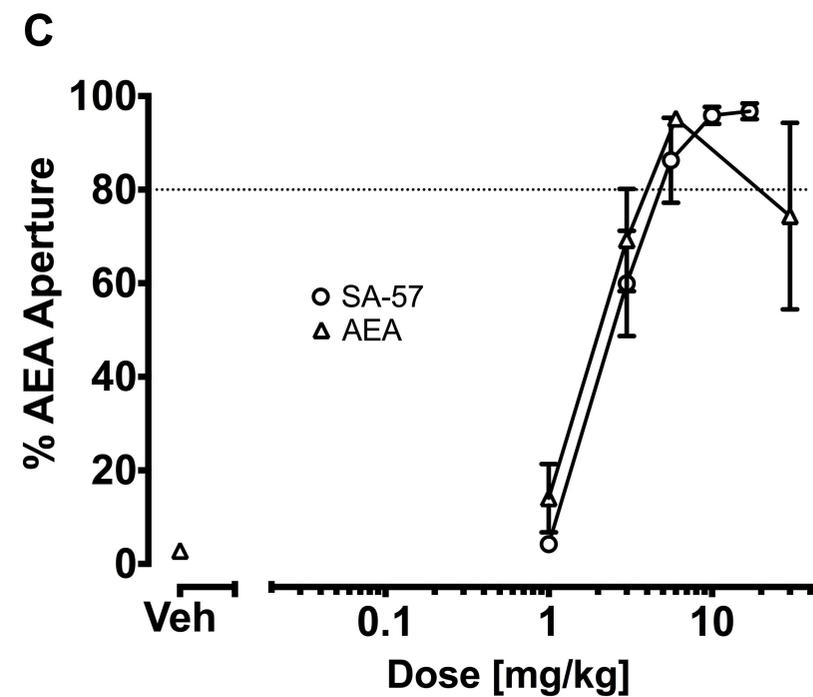
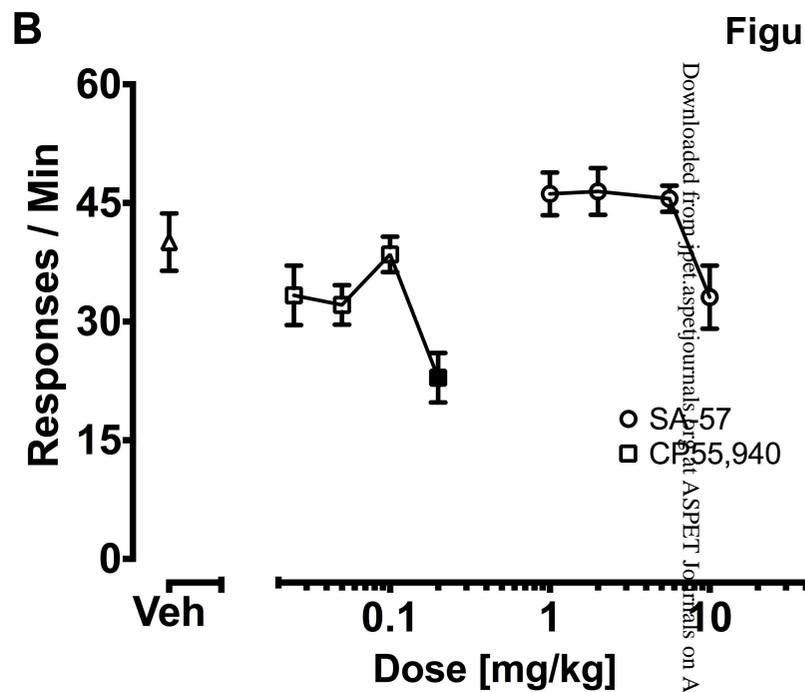
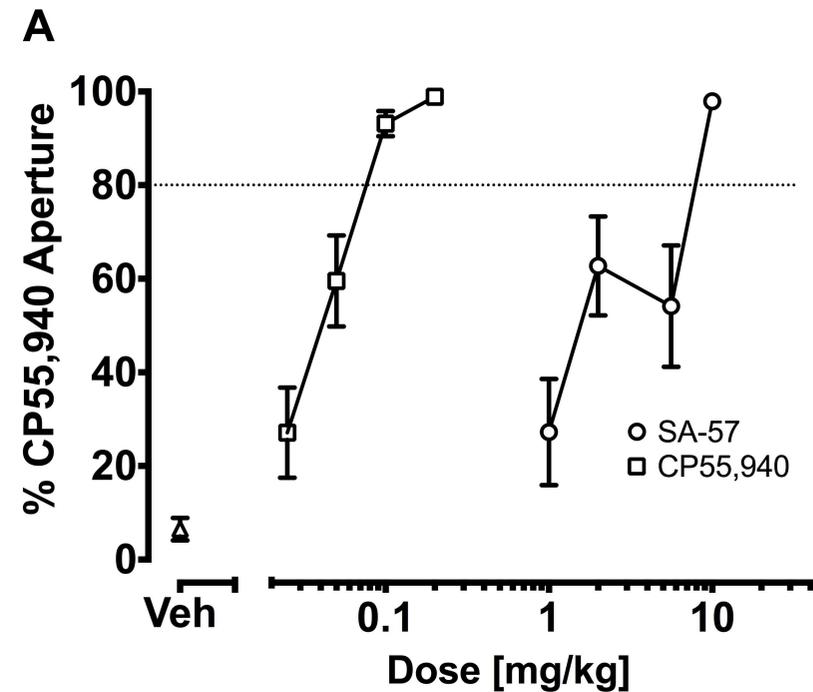
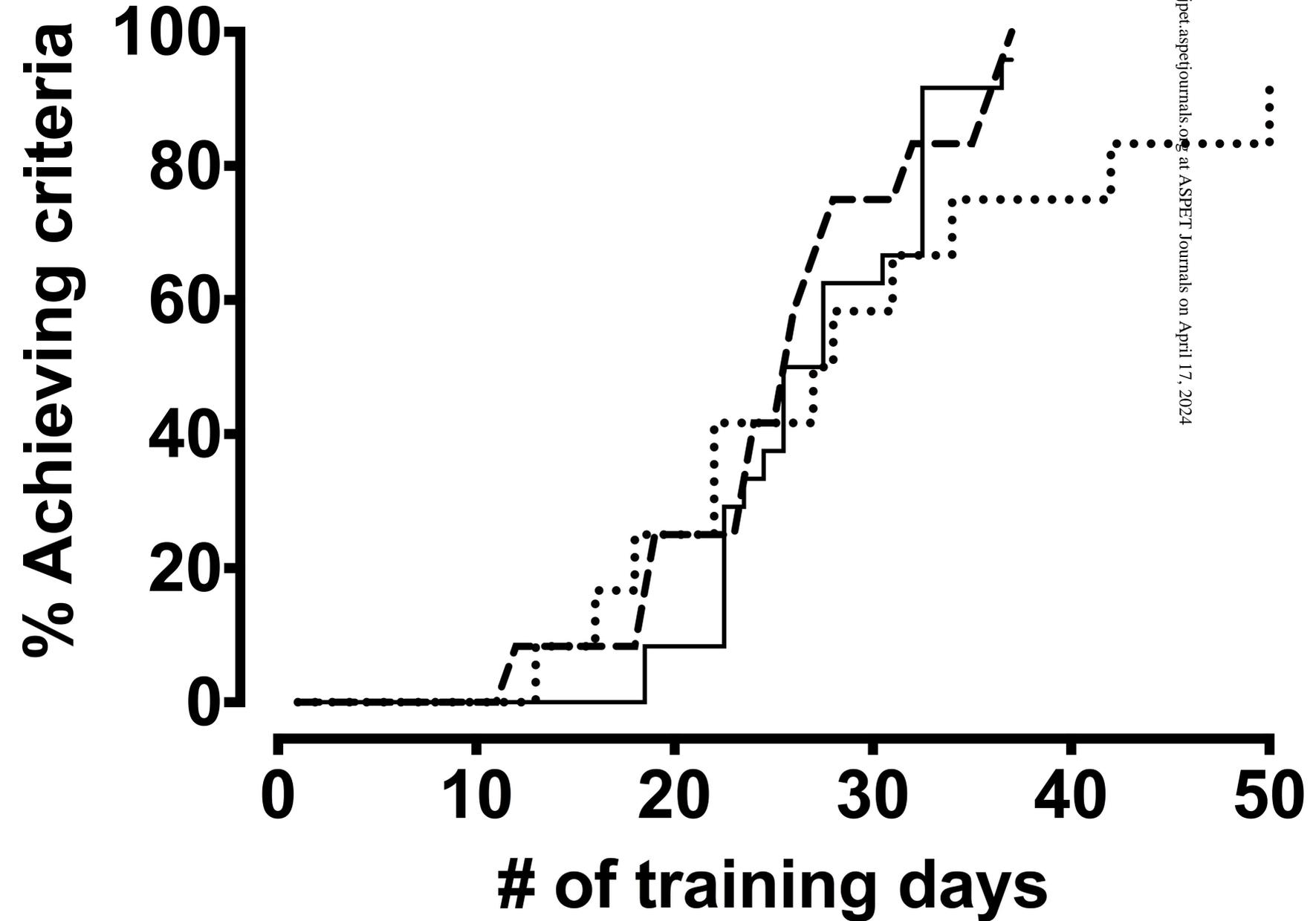


Figure 2

-- CP55,940
— SA-57
... AEA



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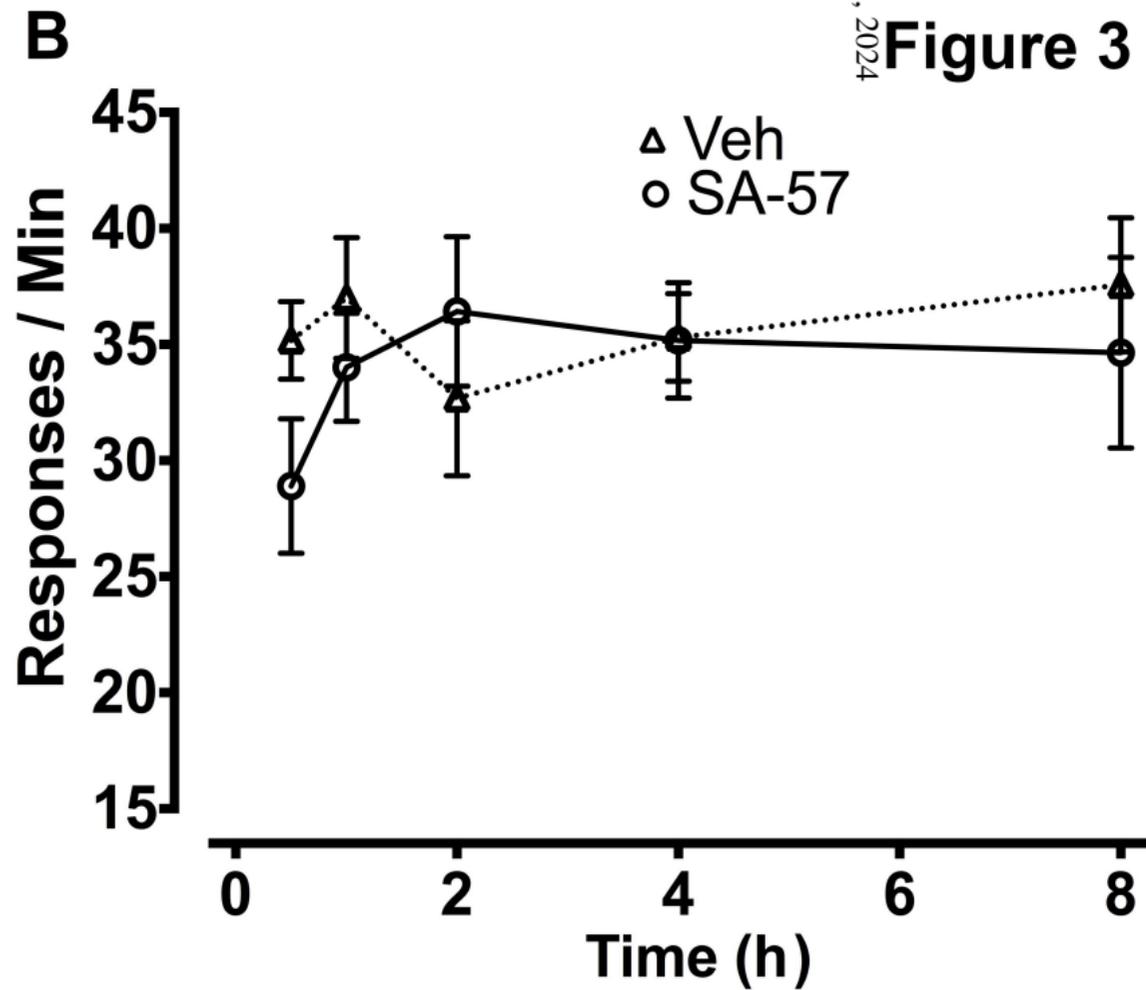
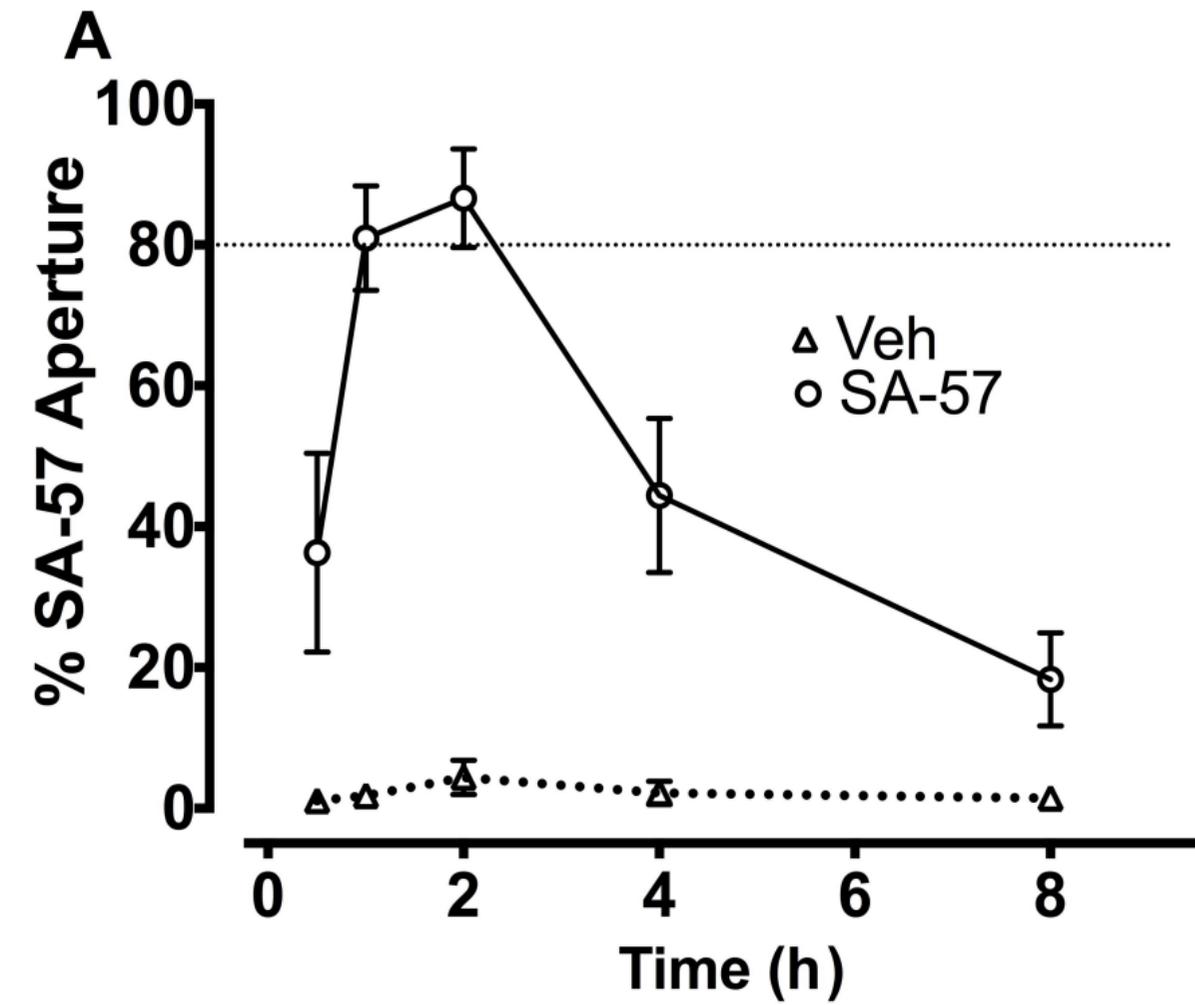


Figure 4

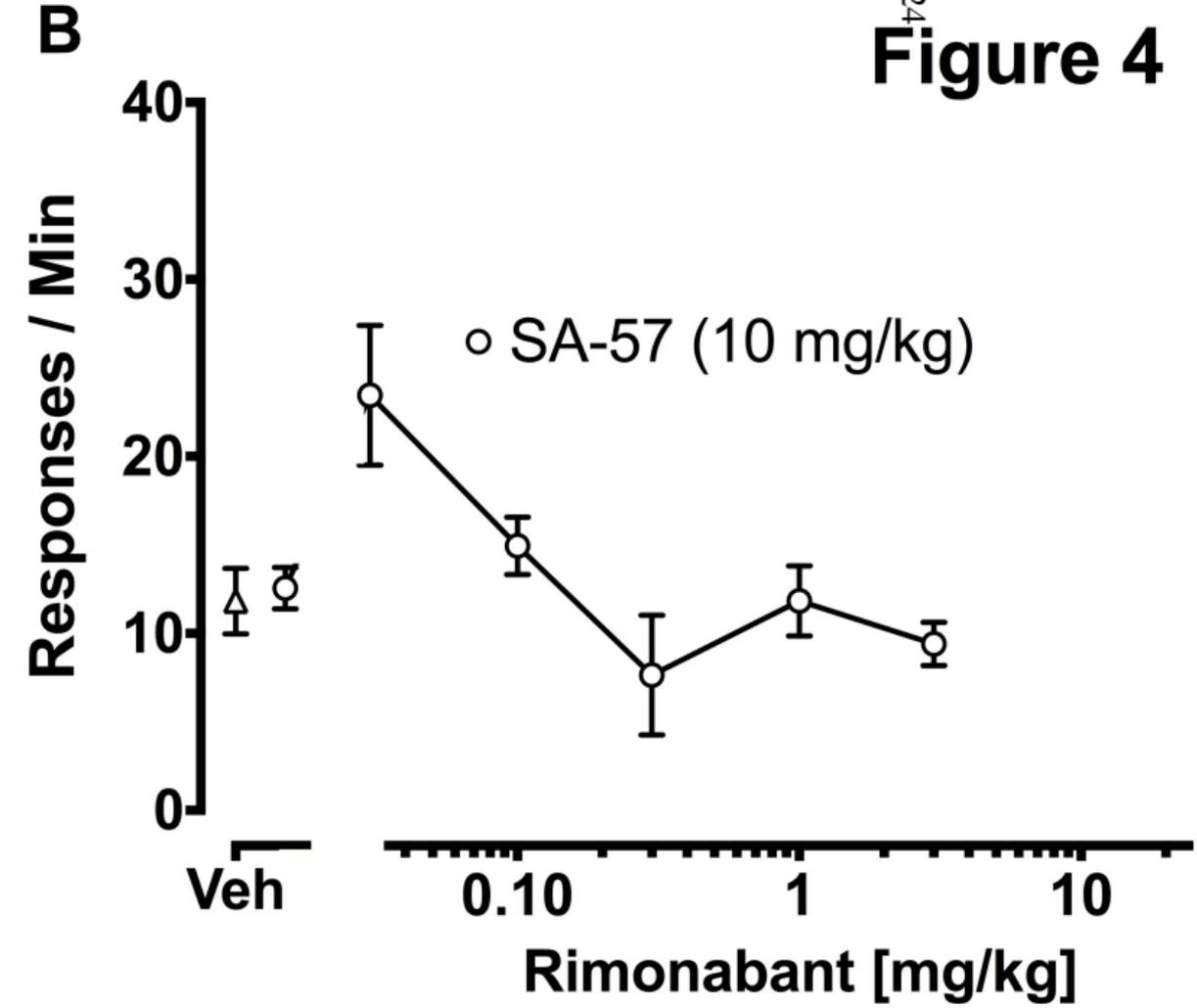
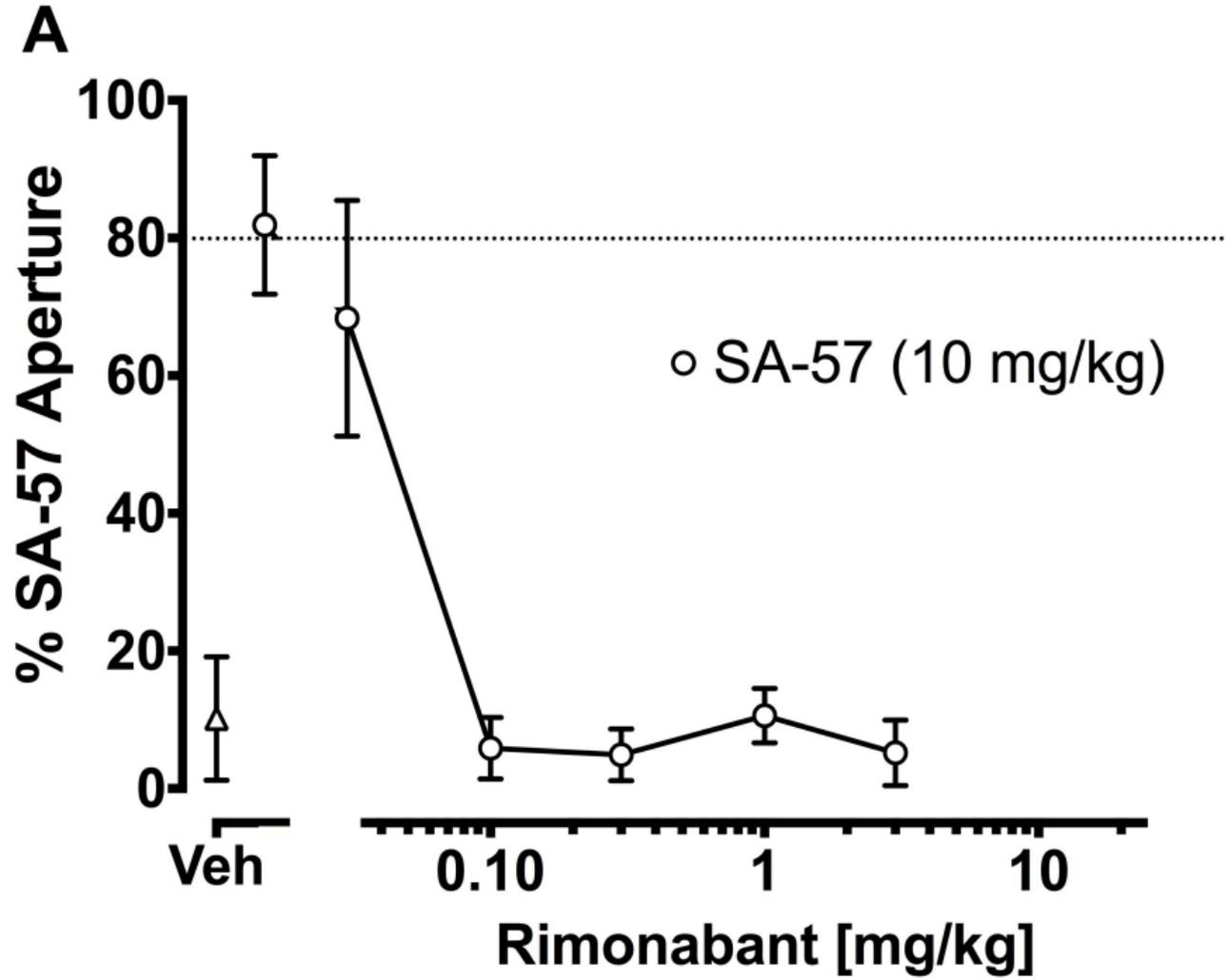
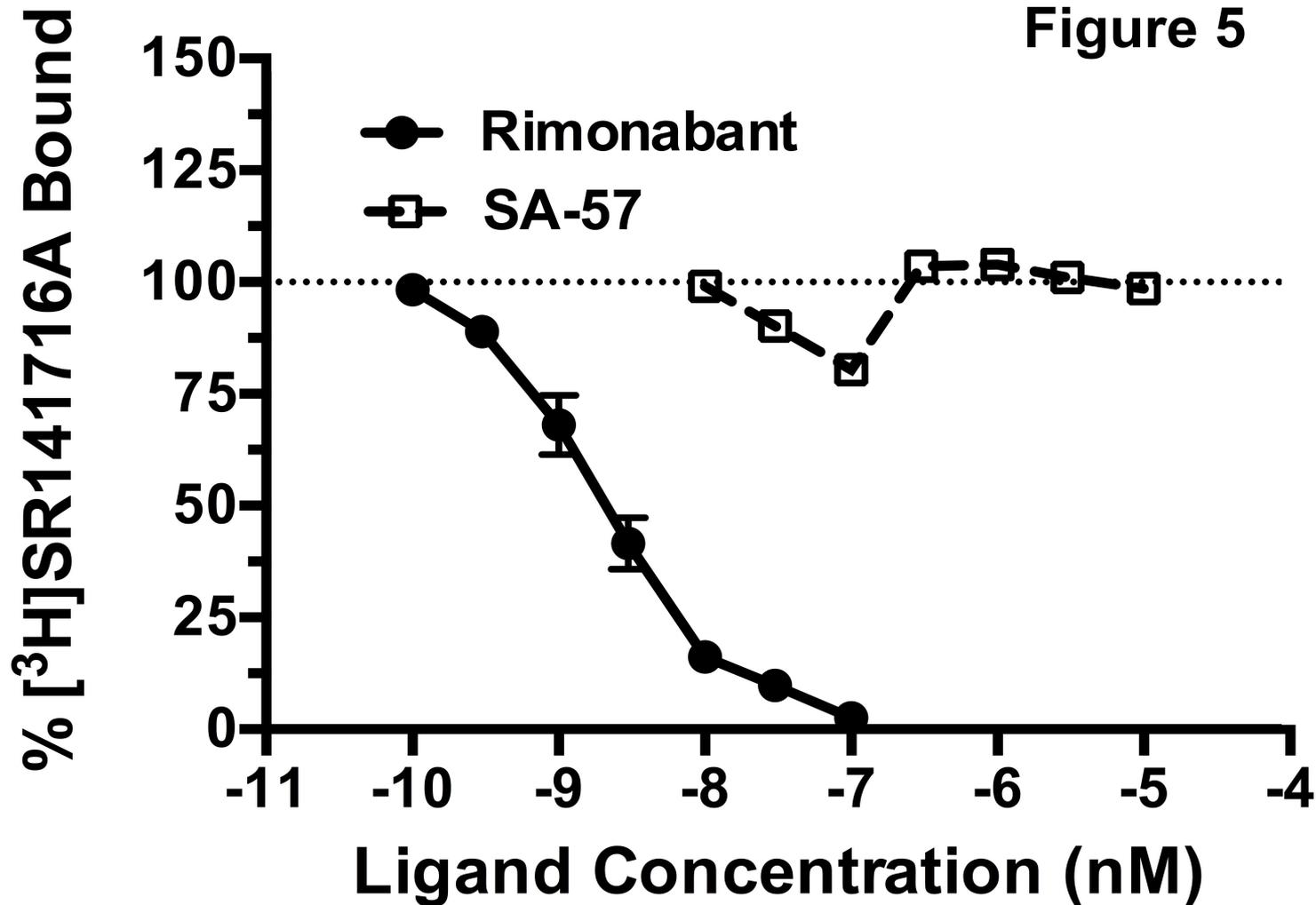
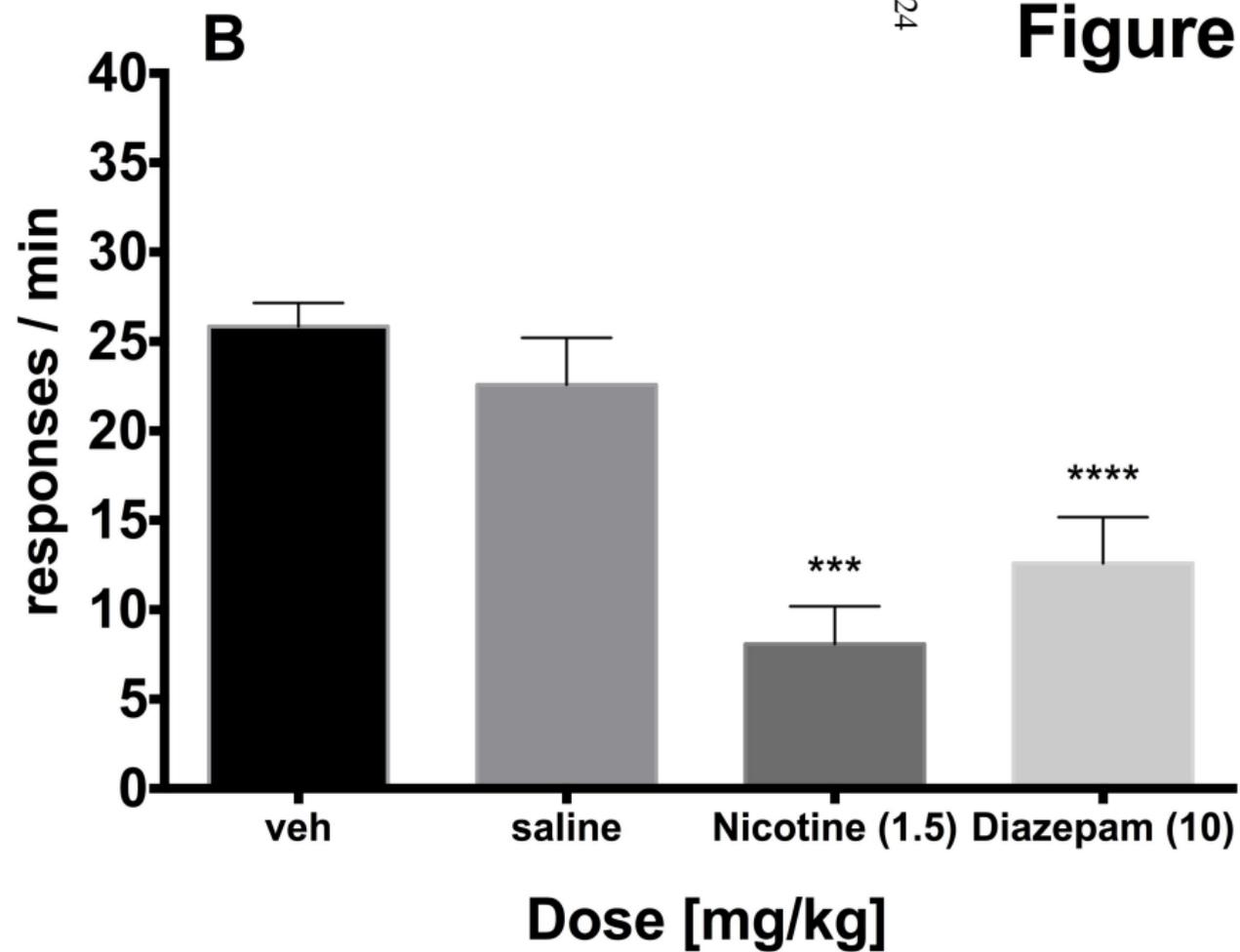
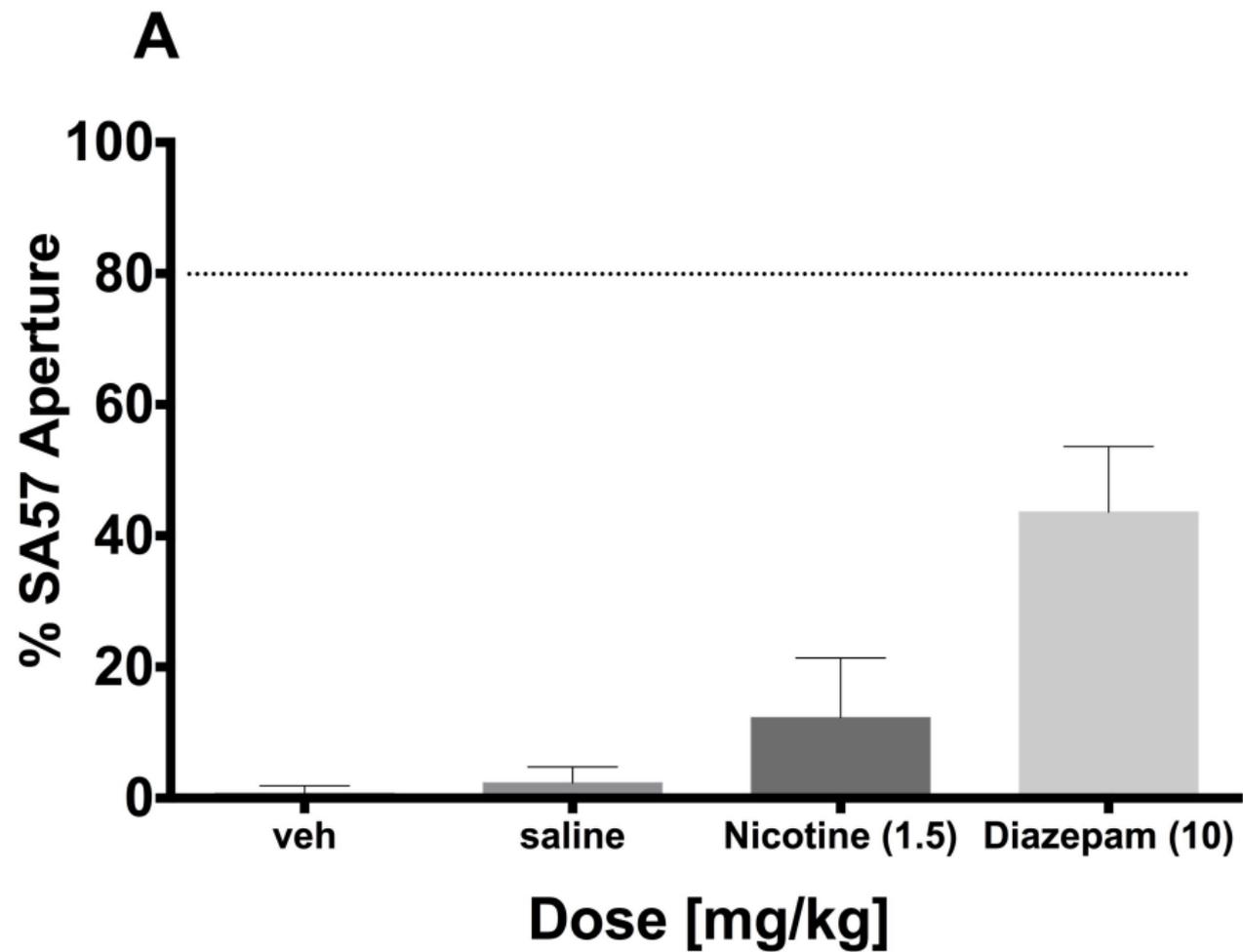
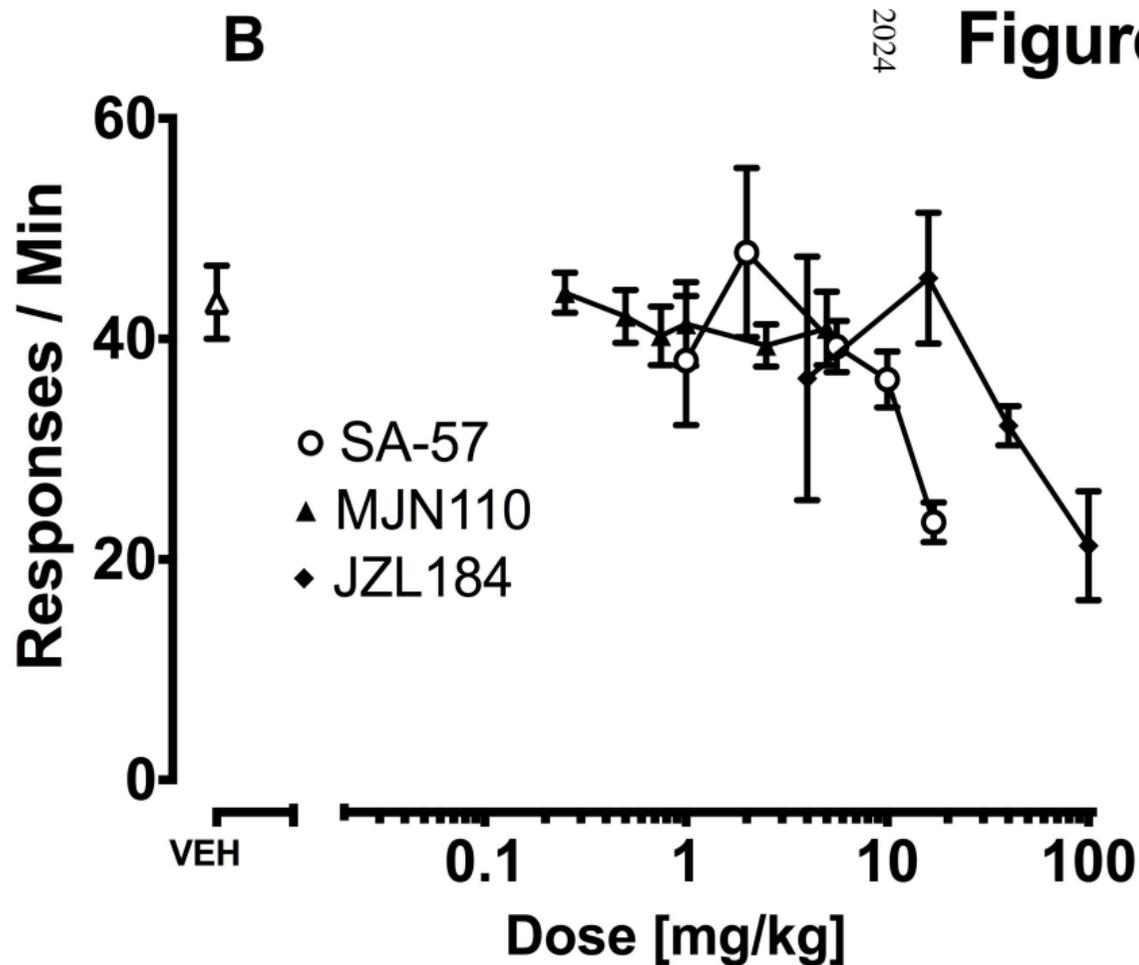
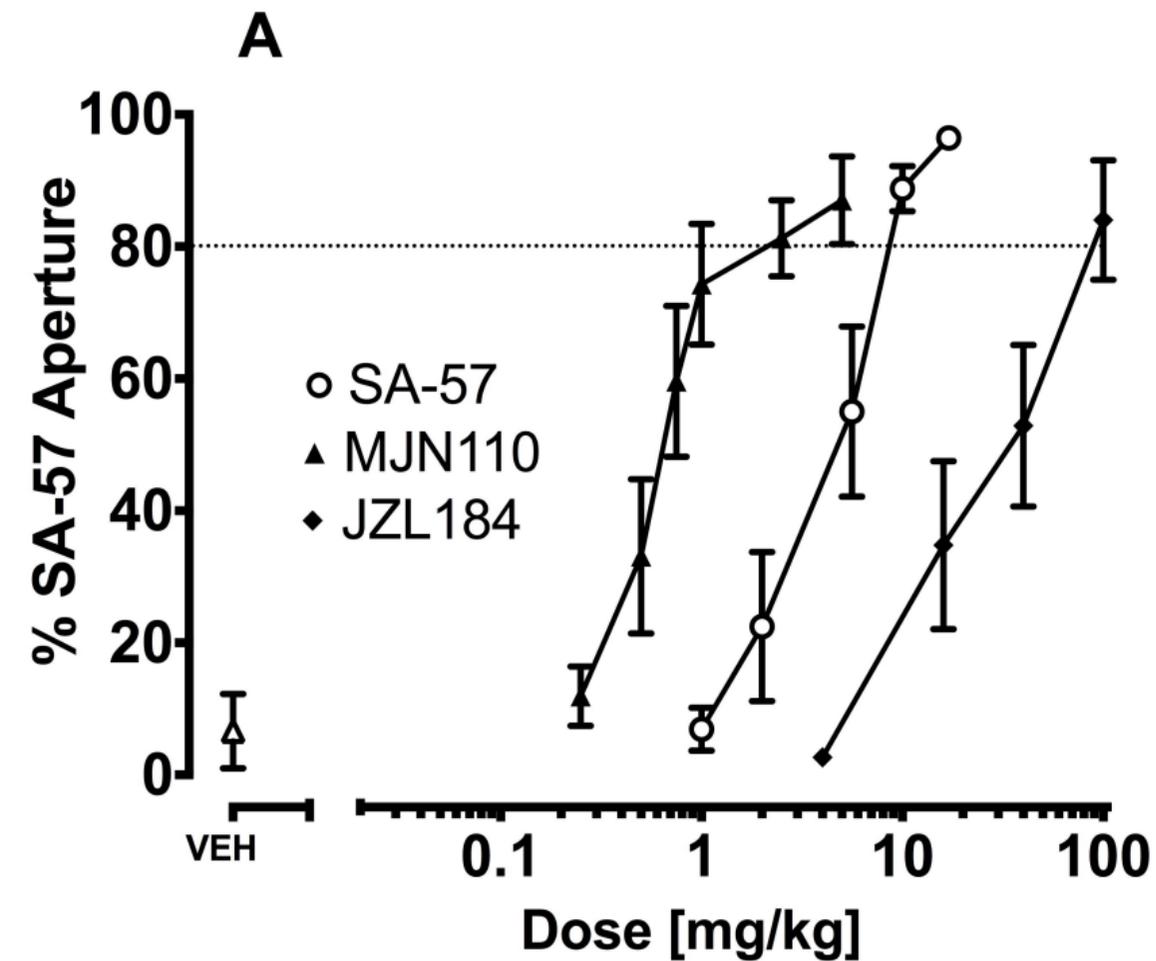


Figure 5







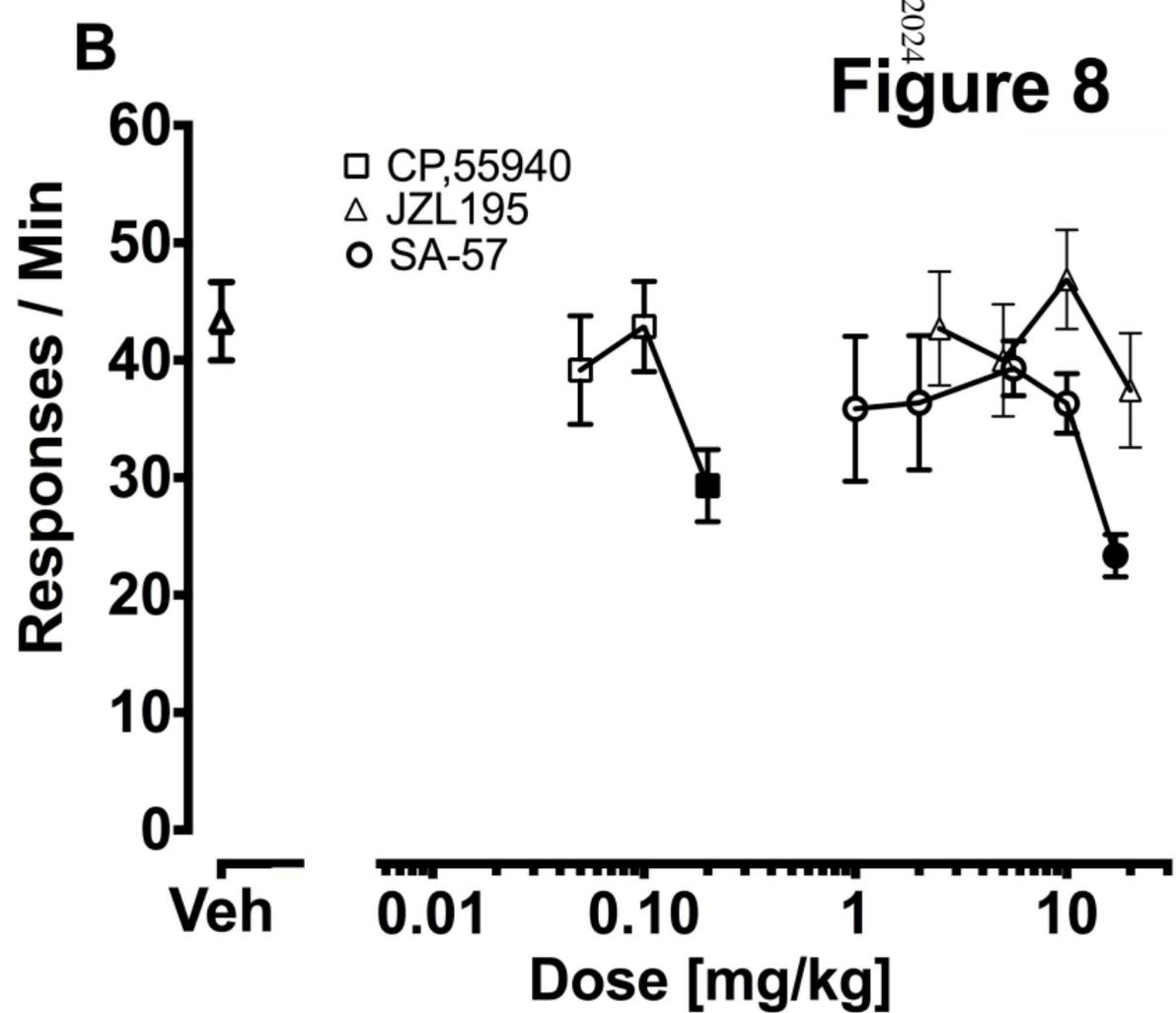
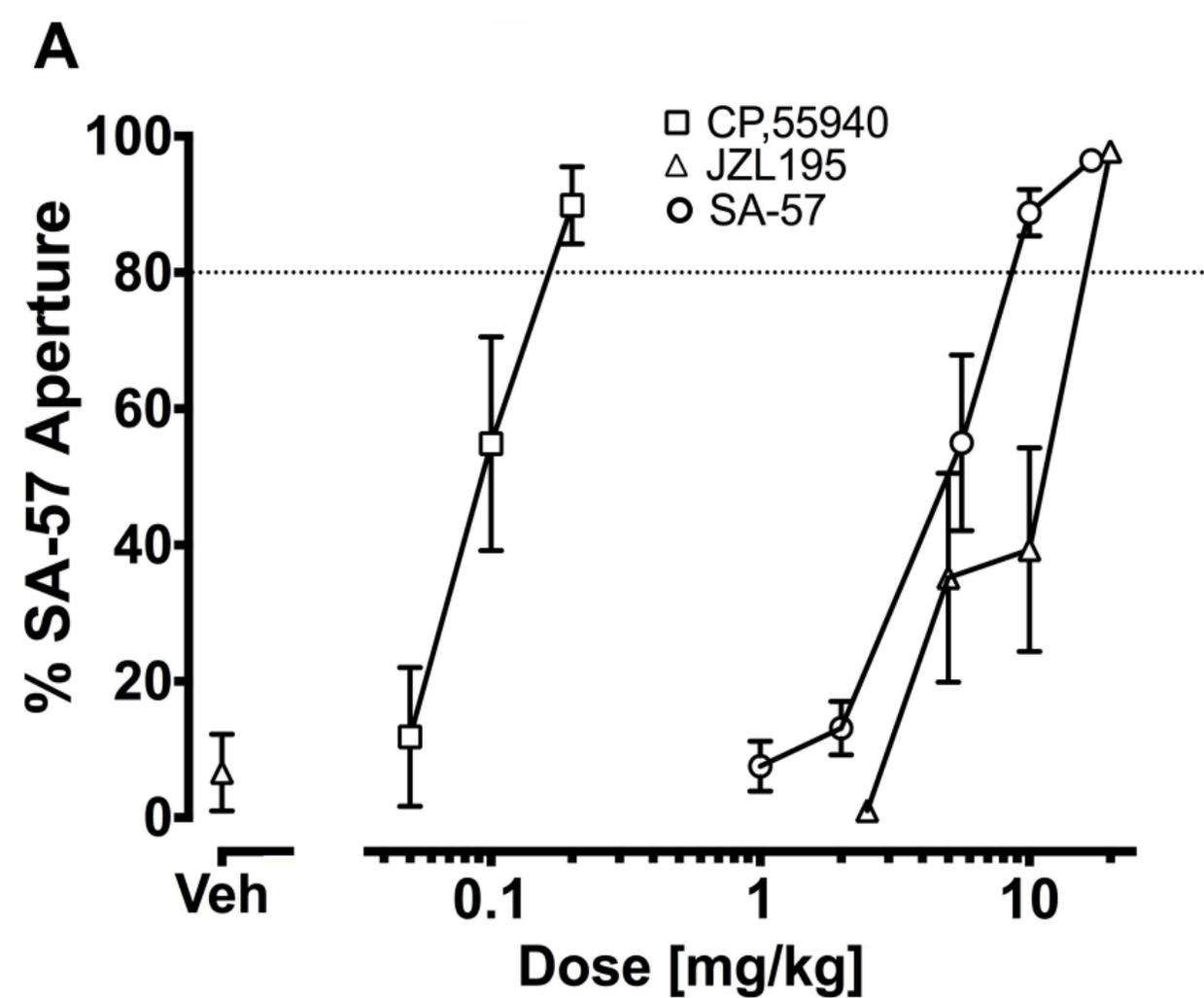


Figure 9

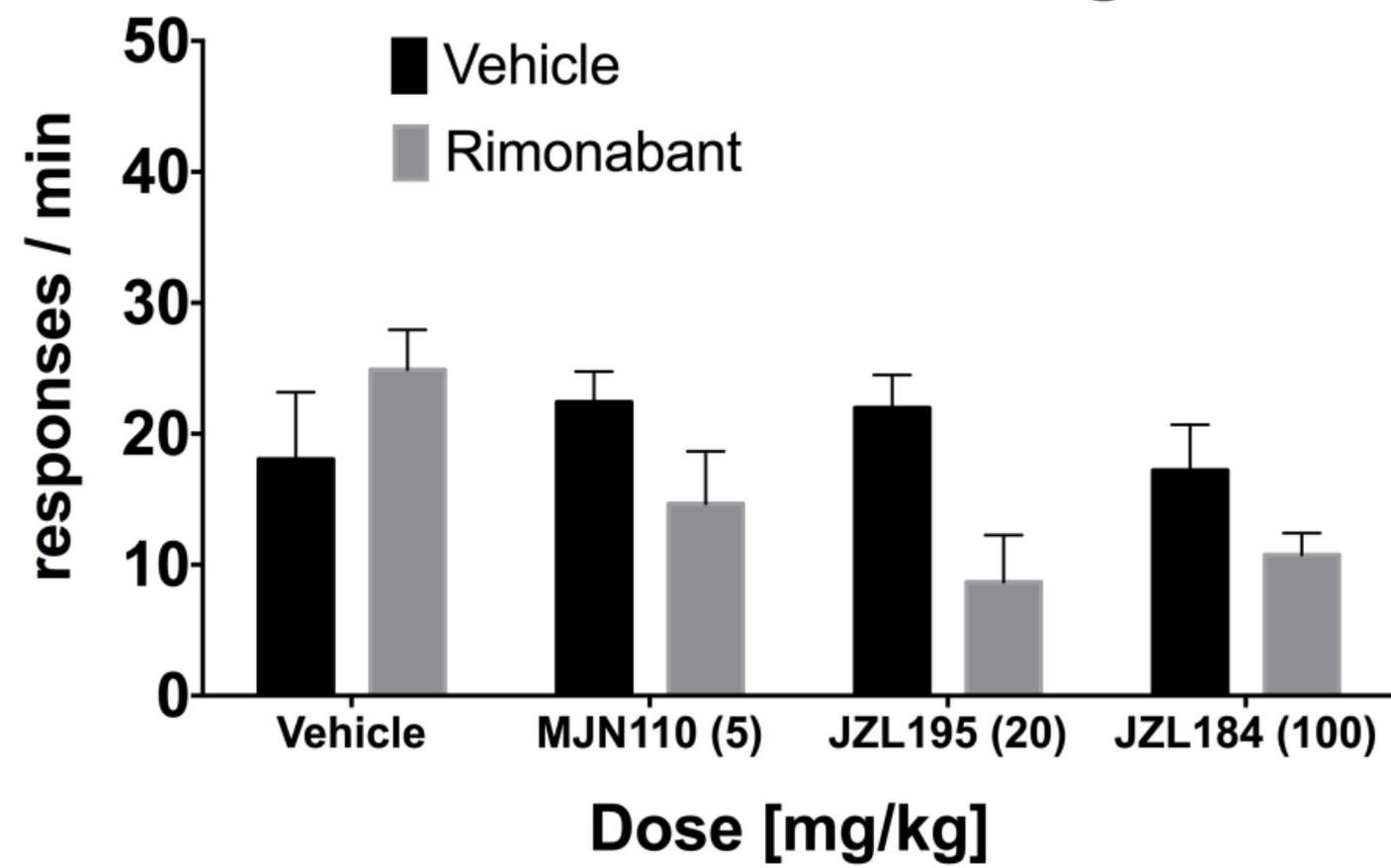
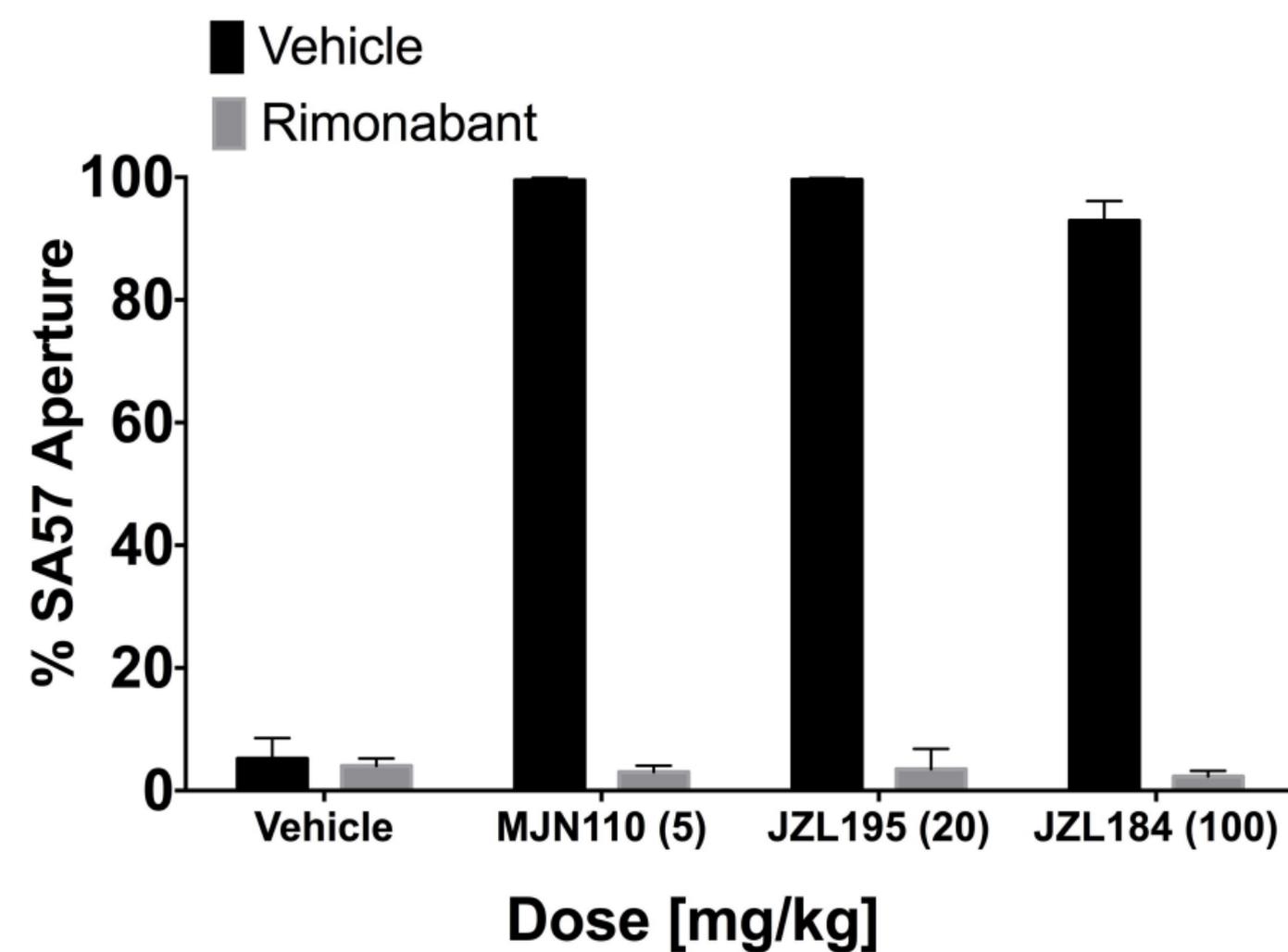


Figure 10

