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Discriminative stimulus properties of 3-substituent rimonabant analogs

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DISCRIMINATIVE STIMULUS PROPERTIES OF 3-SUBSTITUENT RIMONABANT ANALOGS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Abstract

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Cannabinoid agonists (e.g., THC) dose-dependently decrease locomotor activity and body temperature and produce antinociception and catalepsy. Drugs that produce this tetrad of effects within a limited dose range are likely to function as CB1 receptor agonists. A structure activity relationship study from our laboratory investigating analogs of the CB1 antagonist rimonabant revealed that certain alterations in the 3-substituent of rimonabant's pyrazole core conferred agonist-like properties in the tetrad. Interestingly, these effects were present in CB1 -/- mice, and were not reversed by rimonabant in wild-type mice. The present study evaluated two novel 3-substituent rimonabant analogs, O-6629 and O-6658 in the tetrad and drug discrimination, a preclinical model of drug subjective effects that possesses a high degree of pharmacological specificity. Drugs that elicit cannabinergic psychoactive effects in humans are

likely to produce THC-like operant responding in animals trained to discriminate between the interoceptive stimuli produced by THC relative to vehicle.

O-6629 and O-6658 decreased locomotor activity and body temperature and produced catalepsy. O-6629, but not O-6658 produced significant antinociception. However, these drugs differed from THC in regard to the magnitude of tetrad effects observed. These analogs also failed to elicit THC-like discriminative stimulus effects, nor did they antagonize THC's discriminative stimulus in mice discriminating 5.6 mg/kg THC from vehicle. Finally, mice were trained to discriminate 5.6 mg/kg O-6629 from vehicle. O-6658 produced full substitution for O-6629, whereas the cannabinoid agonists THC and anandamide did not. O-6629's discriminative stimulus failed to generalize to rimonabant, cocaine or morphine, whereas WIN 55,212-2 and nicotine evoked partial substitution. These results suggest that these analogs might exert their pharmacological properties through a novel cannabinoid receptor, as has been proposed for WIN 55,212-2 and anandamide. Additionally, O-6629's discriminative stimulus may involve nicotinic acetylcholine or dopaminergic components. Future directions include determining whether the partial substitution observed with nicotine was mediated through a nicotinic mechanism. Tests with chlorpromazine, an antipsychotic that is a false positive in the tetrad, and diazepam, which produces partial substitution for THC's discriminative stimulus through a GABAergic mechanism are also planned.

Discriminative stimulus properties of 3-substituent rimonabant analogs

Cannabinoid pharmacology

The endogenous cannabinoid (hereto referred as endocannabinoid) system is comprised of two primary G-protein coupled receptors, cannabinoid 1 (CB1) and cannabinoid 2 (CB2), and two major endogenous ligands, anandamide and 2-arachidonoylglycerol (2-AG), which activate these receptors. CB1 receptors are located primarily in the brain whereas CB2 receptors are located abundantly in the periphery suggesting functionally different roles for these receptors. Two decades of research have revealed much regarding anandamide's role within the endocannabinoid system. Strong evidence suggests that membrane bound arachidonic acid is likely the precursor for anandamide formation though its synthesis has yet to be completely mapped or understood. Identification of anandamide's degradative pathway has revealed that fatty acid amide hydrolase (FAAH) is primarily responsible for the rapid catabolism of anandamide (Cravatt et al., 1996). Similarly, 2-AG is quickly degraded by a number of enzymes, with monoacylglycrol lipase (MAGL) serving as the primary catabolic enzyme (Long et al., 2009). Development of more stable endocannabinoid ligand analogs (e.g., methanandamide), selective inhibitors of FAAH and/or MAGL (e.g., URB597, JZL184, JZL195), and the development of various cannabinoid-related knockout mice (e.g., CB1 -/-, CB2 -/-, FAAH -/-, MAGL -/-) have facilitated further understanding of the endocannabinoid system and consequently have yielded valuable information regarding its impact on a multitude of biological processes. Accordingly, the endocannabinoid system has become a target of interest for a number of therapeutic purposes, such as management of pain, nausea and emesis. However, the abuse liability and intoxication associated with cannabis greatly limits its accepted therapeutic

use. The following is a brief review of preclinical literature demonstrating the reinforcing efficacy and intoxicating properties of cannabinoids.

Reinforcing properties of cannabinoids

The reinforcing properties of cannabinoids have been especially challenging to demonstrate reliably in a preclinical setting. There have been numerous reports that Δ^9-tetrahydrocannabinol (THC), the primary psychoactive chemical in marijuana (Gaoni & Mechoulam, 1964), fails to maintain self-administration behavior (Kaymakcalan, 1973; Leite & Carlini, 1974; Carney, Uwaydah, & Balster, 1977; Mansbach, Nicholson, Martin, & Balster, 1994), a preclinical model used to examine reinforcing efficacy of drugs (Balster, 1991). However, Tanda et al. (2000) demonstrated that THC does indeed maintain self-administration using a lower dose range per infusion than had typically been studied. In that study, squirrel monkeys with prior experience self-administering cocaine (as reported by Spear, Muntaner, Goldberg, & Katz, 1991) were given a 5 day saline replacement following their most recent cocaine exposure, during which operant responding for an infusion was nearly abolished. Following this extinction period, subjects then were able to respond for 2-4 μg/kg/infusion THC. This low dose infusion, described as comparable to the THC amount inhaled by a human per puff on a marijuana cigarette, readily maintained operant responding. Further, these effects were reversed by pretreatment with selective CB1 antagonist/inverse agonist rimonabant (0.3 mg/kg), whereas responding for cocaine was not influenced by rimonabant, demonstrating CB1 mediation of the reinforcing properties of THC. Once rimonabant pretreatment was ceased, THC-maintained operant responding was reestablished and returned to baseline levels. Although rates of responding for THC and cocaine infusions were comparable, this does not necessarily imply that THC possesses the same degree of reinforcing efficacy as cocaine. A multitude of

procedural parameters, including dose, reinforcement schedule (e.g., FI, FR, second-order) and exposure (e.g., continual vs. limited exposure) can influence self-administration behavior. For these reasons, among others (e.g., subject drug history), determination of relative reinforcement efficacy between drugs can be arduous (Balster, 1991).

A subsequent extension of these studies sought to determine whether previous exposure to cocaine might have influenced results reported by Tanda et al. (2000). Thus, drug naïve squirrel monkeys were employed in a similar paradigm and THC was evaluated for its ability to maintain self-administration behavior. Monkeys were trained to lever press (FR10; 60 s timeout) for an infusion of 4 μg/kg THC, the dose that previously maintained self-administration behavior. This dose did indeed maintain self-administration behavior, and subjects responded at a near maximal rate given the reinforcement contingencies. Once baseline responding for this dose was established, a 5 day vehicle substitution was conducted in between each assessment in the dose effect determination (1-16 μg/kg/infusion). Only 2, 4 and 8 μg/kg/infusion doses resulted in a significantly higher number of infusions per session compared to vehicle. Although rates of responding for THC 16 μg/kg/infusion were not significantly different than vehicle responding, subjects still responded for this high dose and administered a higher cumulative quantity of THC in the high dose condition relative to all other doses tested. In sum, THC is self-administered in humans, as well as non-human primates (under certain conditions), illustrating the reinforcing efficacy of cannabinoids.

The synthetic cannabinoid WIN 55,212-2, a potent full agonist (compared to the plant-derived partial agonist THC) is self-administered in rodents. Both rats (Fattore, Cossu, Martellotta, & Fratta, 2001) and mice (Martellotta, Cossu, Fattore, Gessa, & Fratta, 1998) self-administer WIN 55,212-2 in a dose-dependent manner. Microdialysis results from rats

demonstrate that WIN 55,212-2 self-administration also elevates extracellular dopamine in the shell and core of the nucleus accumbens (Lecca, Cacciapaglia, Valentini, & Di Chiara, 2006). Interestingly, onset of this elevation occurs more rapidly, persists longer and at a greater magnitude in the shell than the core (Lecca et al., 2006). Under extinction conditions, however, dopamine dialysate levels remain unchanged from baseline in both regions, despite the presence of lever-pressing behavior. These microdialysis findings conducted in animals self-administering cannabinoids support the role of the nucleus accumbens shell in the reinforcing properties of drugs with abuse liability (for review, see Di Chiara, 2002).

Intoxicating properties of cannabinoid agonists

The drug discrimination paradigm has been used extensively as a preclinical model for the subjective effects produced by a drug. Based on the principle that certain drugs are capable of serving as discriminative stimuli (i.e., the interoceptive stimuli produced by one drug can be discerned from a non-drug or other drug state), this model typically features an animal trained to make one response (e.g., lever press) after injection of a drug ("training drug") and make a different response (e.g., pressing a different lever) under the presence of vehicle, usually for food reward. Once the animal can reliably discriminate between the discriminative stimulus effects produced by these two distinct states, they can be administered novel compounds to determine whether they elicit discriminative stimulus effects similar to or different than that of the training drug. Drug discrimination research has provided insight on numerous characteristics of drugs, including agonist/antagonist receptor interactions, structure activity relationships, and pharmacokinetics (for review, see Colpaert, 1999).

THC has typically served as the drug of reference for cannabinoid discrimination studies. THC discrimination is believed to model the intoxicating properties produced by marijuana

(Balster & Prescott, 1992) and has been established in numerous species, including rats (Jarbe, Johansson, & Henriksson, 1976; Semjonow & Binder, 1985; Wiley, Barrett, Lowe, Balster, & Martin, 1995b; Burkey & Nation, 1997), non-human primates (Gold, Balster, Barrett, Britt, & Martin, 1992; Wiley, Barrett, Britt, Balster, & Martin, 1993; Wiley, Huffman, Balster, & Martin, 1995a; Wiley, Lowe, Balster, & Martin, 1995b; McMahon, 2009), pigeons (Henriksson, Johansson, & Jarbe, 1975; Jarbe & Hiltunen, 1988; Mansbach, Rovetti, Winston, & Lowe, 1996), and mice (McMahon, Ginsburg, & Lamb, 2008; Vann et al., 2009b; Long et al., 2009).

Several key findings strongly suggest that the discriminative stimulus properties of THC are mediated through CB1 receptor activity. First, the discriminative stimulus of the phytocannabinoid THC is engendered by structurally dissimilar synthetic cannabinoids, including the bicyclic compound CP 55,940 and the indole derivative WIN 55,212-2 (Gold et al., 1992; McMahon et al., 2008). Tests with a number of bicyclic analogs (Compton et al., 1993) and indole derivatives (Vann et al., 2009a) have revealed a strong correlation between CB1 receptor affinity and ability to occasion THC-appropriate responding. Further, non-cannabinoid agents from a variety of distinct pharmacological classes fail to substitute for THC (Browne & Weissman, 1981; Barrett, Wiley, Balster, & Martin, 1995; Wiley et al., 1995a). Lastly, rimonabant readily attenuates THC's discriminative stimulus in all of the aforementioned species (Wiley et al., 1995b; Mansbach et al., 1996; McMahon et al., 2008).

Less clear is the extent to which endocannabinoids may produce THC-like intoxication. Due to rapid degradation of endocannabinoids, it has frequently been difficult to discern to what extent observed differences between THC and endocannabinoid effects may be contributed to pharmacodynamic, rather than pharmacokinetic, factors. Anandamide unreliably substitutes for THC, typically at doses that severely inhibit operant responding (Wiley, Balster, & Martin, 1995;

Solinas et al., 2007b). Several strategies to combat the metabolic instability of endocannabinoids have been developed, including more stable anandamide analogs, such as methanandamide, pharmacologic inhibitors of FAAH and/or MAGL, and transgenic mice lacking enzymes for endocannabinoid hydrolysis (i.e., FAAH -/-). Together, these approaches have offered greater insight into the subjective effects of THC, anandamide, and to a lesser extent, 2-AG.

Methanandamide substitution for THC appears to be, in part, determined by the THC training dose used. Jarbe et al. (1998) reported that methanandamide substitutes for low (1.8 mg/kg, 3.0 mg/kg), but not high (5.6 mg/kg) training doses of THC. This finding has been replicated and reported by others in rats (Burkey & Nation, 1997; Jarbe, Lamb, Lin, & Makriyannis, 2000), non-human primates (McMahon, 2009) and mice (unpublished data from our lab). Additionally, THC fully substitutes for methanandamide in rats (Jarbe, Lamb, Lin, & Makriyannis, 2001; Wiley et al., 2004), demonstrating cross-substitution between the discriminative stimulus effects of THC and an anandamide analog. In drug discrimination, demonstration of cross-substitution is a key indicator that drugs exert intoxicating effects that are mediated through similar or identical mechanisms. Such determinations have provided critical support in classifying both existing and newly developed drugs. Disparities between discriminative stimulus properties of THC and anandamide are frequently attributed to the latter's activity at non-cannabinoid sites, notably transient receptor potential vanilloid type 1 (TrpV1) receptors.

Pharmacological inhibition of FAAH, in conjunction with exogenously administered anandamide, also results in full substitution for THC in rats (Solinas et al., 2007b) and mice (Vann et al., 2009a). In THC-trained FAAH -/- mice, administration of MAGL inhibitor JZL184 resulted in full substitution for THC (Long et al., 2009). In wild-type counterparts, JZL184

produced approximately 50% THC-appropriate responding, suggesting upregulated anandamide levels in FAAH -/- mice were responsible for this discrepancy in JZL184 substitution patterns (Long et al., 2009). This notion was supported following testing with JZL195, an inhibitor of FAAH and MAGL, which produced nearly equivalent levels of THC-appropriate responding in both genotypes (Long et al., 2009).

Recent work from our lab demonstrated that anandamide could serve as a discriminative stimulus in FAAH -/- mice, with anandamide's discriminative stimulus generalizing to THC, but not the fatty acid amide oleamide (Long et al., 2009). In the same study, rimonabant challenge significantly attenuated responding on the anandamide-paired lever for both anandamide and THC, lending further evidence to CB1 mediation of cannabinoid discriminative stimulus effects. Additional unpublished data from our lab collected in anandamide-trained FAAH -/- mice show that indirect elevation of brain 2-AG levels via the MAGL inhibitor JZL184 also produced full substitution for anandamide, highlighting similarities between the subjective effects evoked by endocannabinoids and plant-derived cannabinoids. As such, endocannabinoids appear to be able to elicit THC-like intoxication. Given the mounting interest in endocannabinoid-based therapeutics, more research is needed to understand the cannabimimetic intoxicating potential of such treatment strategies.

Although only compounds that act via CB1 mechanisms substitute for THC, diazepam has been shown to partially engender THC-like responding in rats (Mokler, Nelson, Harris, & Rosecrans, 1986; Barrett et al., 1995; Wiley & Martin, 1999) and rhesus monkeys (Wiley et al., 1995a), but not pigeons (Jarbe & Hiltunen, 1988). This finding appears to be attributable to diazepam activity at GABA, not cannabinoid, receptors, as it was reversed by the GABA antagonist flumanzenil (Mokler et al., 1986), but not rimonabant (Wiley & Martin, 1999). CB1

receptors are frequently co-expressed GABAergic neurons (Tsou, Mackie, Sanudo-Pena, & Walker, 1999), providing a tenable explanation for this finding.

A number of pharmacological agents can modify THC's discriminative stimulus. The μ-opioid agonists heroin and morphine, but neither the δ-opioid agonist SNC-80 nor the k-opioid agonist U50-488, potentiated THC's discriminative stimulus in rats (Solinas, Zangen, Thiriet, & Goldberg, 2004; Solinas & Goldberg, 2005a). This effect was reversed with the μ-opioid antagonist naltrexone, but not by δ- or k-opioid antagonists (Solinas & Goldberg, 2005a). Additionally, naltrexone shifted the THC dose effect curve rightward, an effect that did not extend to δ- or k-opioid antagonists (Solinas & Goldberg, 2005a). Since none of these opioid agonists produced meaningful THC-appropriate responding when given alone, it appears that μ-opioid receptors can modulate the intoxicating properties of THC.

Similarly, cholinergic and D2 dopaminergic activation can enhance THC's discriminative stimulus in rats. Both nicotine and pilocarpine (nicotinic and muscarinic acetylcholine agonists, respectively) potentiate THC's discriminative stimulus, but fail to substitute on their own (Solinas et al., 2007a). Likewise, general dopamine agonists (cocaine, amphetamine) and D2 agonists (quinpirole and apomorphine) also produced a similar pattern (i.e., potentiation, but lack of substitution) that was not seen with D1 agonists (Solinas, Tanda, Wertheim, & Goldberg, 2010). In sum, substitution for THC is positively correlated with CB1 receptor affinity, reversed by CB1 antagonists, and non-cannabinoids do not fully substitute for THC, supporting the precise pharmacological specificity typically offered in drug discrimination experiments. However, activity at several other receptors has been shown to modify or produce partial substitution for THC's discriminative stimulus, reflecting the complex interactions between the endocannabinoid system and a multitude of other neurochemical systems.

Rimonabant

The endocannabinoid system's known involvement in many physiological processes, including appetite, reward, and cognition, coupled with its interactions with other neurochemical systems suggest a vast range of potential therapeutic applications for a selective CB1 antagonist. In 1994, scientists from Sanofi-Aventis reported on the development of the first CB1 antagonist, SR141716A (5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; hereto referred as rimonabant), which displayed approximately 1000-fold selectivity for CB1 over CB2 receptors (Rinaldi-Carmona et al., 1994). In this seminal report, rimonabant dose-dependently antagonized cannabinoid-induced changes on mouse van deferens contractions and adenylate cyclase activity in rat brain membranes, and reversed characteristic cannabinoid behavioral effects, including antinociception, hypothermia, and ring immobility.

While rimonabant is typically referred to as a selective CB1 antagonist (i.e., possessing no intrinsic activity at the receptor), there is a body of literature suggesting that rimonabant functions as an inverse agonist (i.e., exerting pharmacological effects opposite of agonist activity at the receptor). The first behavioral evidence supporting this theory came from Compton et al. (1996), who demonstrated that rimonabant stimulated locomotor activity at higher doses ($\geq$ 3 mg/kg, i.v.). Rimonabant-induced stimulation of locomotor activity could have been due to a number of factors, including inhibition of endocannabinoid tone or via activity through an unknown non-CB1 mechanism (Compton et al., 1996). In vitro work also substantiated claims of rimonabant's inverse agonist activity as demonstrated by adenylyl cyclase activity (Meschler, Kraichely, Wilken, & Howlett, 2000) and effects on basal [^{35}S]GTPγS binding (Landsman, Burkey, Consroe, Roeske, & Yamamura, 1997; Sim-Selley, Brunk, & Selley, 2001).

An investigation by Bass and Martin (2002) supported the hypothesis that inverse agonist properties of rimonabant were not attributable to its ability to stimulate locomotor activity. A series of rimonabant analogs that displayed inverse agonist activity at CB1 receptors in [^{35}S]GTPγS binding assays were incapable of stimulating locomotor activity relative to vehicle controls. Additionally, some rimonabant analogs with low CB1 affinity did not function as inverse agonists as CB1 receptor inverse agonists, but did increase locomotor activity (Bass et al., 2002).

Subsequent investigations of rimonabant have vastly aided understanding and manipulation of the endocannabinoid system. Indeed, utilization of rimonabant as a mechanistic tool for exploration of the endocannabinoid system has been responsible for many critical findings that form the underpinnings of cannabinoid pharmacology. The breadth and depth of such findings are too great as to aspire to review thoroughly. Thus, the following review of rimonabant's pharmacological properties will focus on several key areas: 1) rimonabant as a mechanistic tool to characterize cannabinoid dependence and withdrawal, and data implicating CB1 receptor antagonism for the treatment of 2) substance abuse disorders and 3) obesity.

Cannabinoid withdrawal

Prior to the synthesis of a selective CB1 receptor antagonist, reports of spontaneous cannabinoid withdrawal were sparse (McMillan, Dewey, & Harris, 1971; Kaymakcalan, Ayhan, & Tulunay, 1977; Beardsley, Balster, & Harris, 1986), while others failed to observe withdrawal (Leite & Carlini, 1974; Harris, Waters, & McLendon, 1974). This mild spontaneous withdrawal syndrome (relative to other drugs of abuse such as ethanol or cocaine) is likely due to the relatively long half-life of THC and other cannabinoids (Compton, Dewey, & Martin, 1990). Shortly after the development of rimonabant, it was reported that it precipitated withdrawal in

rats following chronic treatment with THC (Aceto, Scates, Lowe, & Martin, 1995; Tsou, Patrick, & Walker, 1995). Tsou et al. (1995) observed a withdrawal syndrome characterized by disorganized motor behavior, frequent, but not repetitive, wet dog shakes and forepaw flutters, as well as increases in grooming behavior, horizontal ambulation and rearing (Tsou et al., 1995). Other behavioral signs indicative of withdrawal, such as head shakes, facial rubbing, and stretching have been noted as well (Aceto et al., 1995).

Since these initial findings were reported, rimonabant precipitated withdrawal has been demonstrated in a number of species and has been extended to other cannabinoids. Indeed, both exogenous (e.g., WIN 55,212-2; Aceto, Scates, & Martin, 2001) and endogenous (e.g., AEA; Costa, Giagnoni, & Colleoni, 2000) cannabinoids have been shown to produce physiological dependence, as indicated by the presence of withdrawal symptoms upon removal of chronic cannabinoid administration or precipitated withdrawal following rimonabant administration. Also of note is the ability of direct or indirect activation of the endocannabinoid system to alleviate withdrawal symptoms. Treatment with either THC or inhibitors of endocannabinoid hydrolysis during rimonabant-precipitated withdrawal have been shown to reduce the expression of withdrawal symptoms, such as paw flutters, in mice (Lichtman, Fisher, & Martin, 2001; Schlosburg et al., 2009). Accordingly, rimonabant has served as a valuable pharmacological tool in determining the ability of cannabinoids to produce physiological dependence. Further, this model provides a means for evaluating putative pharmacotherapies for the treatment of cannabis dependence.

Discriminative stimulus properties of rimonabant

One significant objective of studying cannabinoid dependence is to develop strategies for treatment of the withdrawal symptomology. Though early attempts to train rimonabant were

largely unsuccessful (Wiley, 1999), McMahon and France (2003) established rimonabant as a discriminative stimulus in rhesus monkeys chronically treated with THC (1.12 mg/kg/day). In this regard, the discriminative stimulus effects produced under this dosing regiment arguably serve as another avenue to model cannabis withdrawal, and consequently evaluate therapeutic agents for its treatment. Monkeys were trained to press one lever following administration of rimonabant (1 mg/kg) and press another lever following vehicle to avoid an electric foot shock. Once stimulus control was established, it was demonstrated that rimonabant's discriminative stimulus was dose-dependent, and discontinuation of chronic THC treatment engendered rimonabant-like responding alone. This effect was reversed once THC treatment was resumed. Further, THC (in addition to the chronic regimen) dose-dependently attenuated rimonabant's discriminative stimulus, whereas cocaine and ketamine failed to substitute for rimonabant, demonstrating the pharmacological specificity of rimonabant's discriminative stimulus.

In a subsequent study, it was shown that the potent synthetic cannabinoid, CP 55,940, also fully blocked rimonabant's discriminative stimulus, whereas WIN 55,212-2, another potent synthetic cannabinoid, only did so partially (Stewart & McMahon, 2010). Additionally, the α2-adrenergic agonist clonidine, but not the $GABA_A$ positive allosteric modulator, diazepam, partially attenuated rimonabant's discriminative stimulus, suggesting that clonidine may be a viable treatment option for the subjective dysphoria present in cannabis withdrawal (Stewart & McMahon, 2010). Indeed, a study conducted in individuals meeting criteria for cannabis dependence found that a combination of THC and lofexidine (another α2-adrenergic agonist) significantly attenuated a number of subjective (e.g., marijuana craving, irritability, quality of sleep) and objective (e.g., latency to fall asleep, percentage of time spent sleeping) withdrawal measures during a period of cannabis abstinence (Haney et al., 2008). This combination appeared

more efficacious relative to either drug alone or vehicle and elicited a "neutral" feeling for the subjective effects rating, whereas individuals reported liking the subjective effects produced by THC alone. Together, these findings promote further investigations of α2-adrenergic agonists for their therapeutic potential, either alone or as an adjunctive treatment for cannabis withdrawal.

Rimonabant has also been trained as a discriminative stimulus using discriminative taste aversion procedure (Jarbe, Harris, Li, Liu, & Makriyannis, 2004; Jarbe, Li, Vadivel, & Makriyannis, 2008). In contrast with more traditionally utilized operant based drug discrimination paradigm, discriminative taste aversion is based upon Pavlovian conditioning principles. In this model, rats were treated with 5.6 mg/kg rimonabant, given access to drinking water, and then treated with lithium chloride, a noxious stimulus that markedly decreased drinking behavior (i.e., unconditioned response). During another pairing session, subjects were treated with saline, given access to drinking water, and then received another injection of saline. Future rimonabant administration resulted in significantly less drinking behavior compared to saline. Thus, rimonabant served as a conditioned stimulus and could dose-dependently elicit the same effect as the unconditioned stimulus, lithium chloride (Jarbe et al., 2004). AM251, a CB1 antagonist, produced a rimonabant-like decrease drinking behavior, whereas THC and the CB2 antagonist SR144528 produced levels of drinking similar to saline (Jarbe et al., 2004). When co-administered with rimonabant, THC dose-dependently attenuated the decrement in drinking behavior (Jarbe et al., 2004). Collectively, these results indicate that rimonabant can exhibit cannabinoid-mediated discriminative stimulus effects following certain methodological manipulations.

Therapeutic potential for non-cannabinoid drug abuse disorders

CB1 receptors are located abundantly throughout the central nervous system and are frequently co-localized with other receptors. Particularly noteworthy is that CB1 receptors are prevalent among brain areas associated with reward, such as the nucleus accumbens. In vivo microdialysis has demonstrated that anandamide and methanandamide (a metabolically stable analog of anandamide) elevate levels of DA in the shell of the nucleus accumbens (Solinas et al., 2006). In the same study, inhibition of FAAH (via URB597 pretreatment) potentiated anandamide-induced release of DA, although URB597 had no effects on DA release when given alone. Rimonabant, but not the TrpV1 antagonist capsazepine, reversed these effects, strongly implicating CB1 receptor activity in reward processes. Thus, the endocannabinoid system likely exerts modulatory effects over systems regulating drug abuse behavior and may attenuate reward processes attributed to other abused drugs.

A number of studies have suggested a potential role for rimonabant in nicotine cessation therapy. For instance, a dose effect determination of rimonabant (0.03-1 mg/kg) pretreatment on nicotine self-administration (0.03 mg/kg/infusion) in rats revealed a dose-and trial-dependent decrease in responses on the active lever and number of infusions (Cohen et al., 2002). The low dose of rimonabant (0.03 mg/kg) failed to alter self-administration behavior. The 0.1 mg/kg dose of rimonabant decreased responding on the active lever and infusions on the second trial, but not the first, whereas 0.3 and 1 mg/kg did so on both the first and second trials. However, presses on the inactive lever were significantly decreased by rimonabant on trial 1 (1 mg/kg) and 2 (0.3 mg/kg), suggesting potential motor disruptions at these higher doses. Further, in the same study, conducted by scientists at Sanofi-Aventis, rimonabant failed to block nicotine's discriminative stimulus in nicotine-trained rats (Cohen et al., 2002). Interestingly, nicotine substitution for d-amphetamine was reversed by rimonabant (3 mg/kg). *In vivo* microdialysis revealed significant

attenuation of nicotinic enhancement of extracellular dopamine in both the shell and bed nucleus of the stria terminalis of the nucleus accumbens (Cohen et al. 2002). These results suggest that rimonabant might attenuate some of the reinforcing, but not subjective, effects of nicotine through a dopaminergic mechanism.

Rimonabant (3 mg/kg) also has been shown to block the development of nicotine conditioned place preference (CPP), as well its expression in rats. Acute rimonabant only abolished expression of nicotine CPP 24h removed from the last conditioning trial (Forget, Hamon, & Thiebot, 2005). Conversely, chronic rimonabant treatment blocked expression of nicotine CPP 2-3 weeks following conditioning. Locomotor activity was decreased during all nicotine conditioning trials in rats pretreated with rimonabant, relative to nicotine alone, thus clouding interpretation of these findings. Further, the anhedonic properties associated with rimonabant (e.g., Beyer et al., 2010; Horder, Harmer, Cowen, & McCabe, 2010) are another important consideration when evaluating its effects in CPP experiments, especially given the disparities between acute and chronic rimonabant treatment on expression of nicotine CPP.

One factor commonly attributed to nicotine relapse is the weight gain users frequently experience during quit attempts (Williamson et al., 1991). Existing treatment strategies are either only temporarily effective (e.g., buproprion; Hurt et al., 1997; Jorenby et al., 1999) or inconsistent (e.g., nicotine replacement therapy; Perkins, 1993) in attenuating weight gain induced by tobacco abstinence. Coupled with its noted effects on appetite, rimonabant's efficacy in preclinical models of nicotine reward and dependence made it an attractive clinical target for treatment of nicotine dependence. Indeed, rimonabant progressed to Phase III clinical trials to evaluate its efficacy and safety alone (studies with rimonabant and tobacco use; STRATUS) and as an adjunct to nicotine replacement therapy (Rigotti, Gonzales, Dale, Lawrence, & Chang,

2009). Given alone, 20 mg rimonabant daily facilitated tobacco abstinence during a 10 week treatment period, with 36% of patients remaining abstinent relative to those receiving 5 mg rimonabant (20.2%) or placebo (20.6%). Nausea was reported in nearly 16% of the 20 mg group, but only in 9.2% of the placebo group. Moreover, a higher attrition rate was noted in those taking 20 mg rimonabant compared to placebo (6.9 vs. 3.8%, respectively). Rimonabant was also more effective in preventing relapse in combination with a nicotine patch (39.0%) versus rimonabant alone (21.3%, Rigotti et al., 2009). Weight gain at the end of treatment was negligible (< 0.5 kg) for both treatment groups and a similar number of adverse incidents were reported between groups (Rigotti et al., 2009). Specifically, depression and anxiety were noted in 4.2 and 5.8% of patients, respectively, with approximately 2% of patients discontinuing treatment due to the severity of depression or anxiety experienced. One patient (out of 754) attempted suicide (Rigotti et al., 2009).

Results from clinical studies suggested rimonabant to be moderately efficacious in treating nicotine dependence, especially in preventing post-abstinence weight gain. Unfortunately, the propensity of rimonabant to produce nausea and more importantly, psychiatric symptoms, dampened enthusiasm for this treatment strategy. By the time Rigotti and colleagues (2009) published their findings, rimonabant had already been withdrawn from all markets by Sanofi-Aventis, as discussed later. Nonetheless, given the importance of developing novel treatment strategies for nicotine dependence, the benefit to risk ratio of rimonabant arguably warrants further discussion and exploration of CB1 antagonists as clinical targets.

Preclinical data also suggest a role for CB1 antagonists in the treatment of several other substance abuse disorders. For instance, opiate self-administration in rats is frequently attenuated by rimonabant (Braida, Pozzi, Parolaro, & Sala, 2001; Caille & Parsons, 2003; De Vries,

Homberg, Binnekade, Raaso, & Schoffelmeer, 2003; Solinas, Panlilio, Antoniou, Pappas, & Goldberg, 2003). Caille and Parsons (2006) utilized in vivo microdialysis and self-administration techniques to investigate neural mechanisms that may be responsible for this observation. Two key brain regions known to influence opioid reward are the nucleus accumbens and its projections to the ventral pallidum. Opiate administration inhibits neuronal GABA activity, consequently reducing GABA efflux in the ventral pallidum. Moreover, heroin self-administration is blocked by pharmacological enhancement of GABA in the ventral pallidum (Bardo, 1998), providing evidence that agents that restore GABA activity might be clinically relevant for treatment of opioid dependence.

In vivo microdialysis revealed the rimonabant dose-dependently reversed morphine-induced decreases in ventral pallidum GABA efflux, but did not alter interstitial DA increases in the nucleus accumbens shell (Caille & Parsons, 2006). Furthermore, rimonabant had no effect on cocaine-induced changes to GABA or DA in the ventral pallidum or nucleus accumbens shell, respectively. Bilateral administration of rimonabant in the nucleus accumbens, but not ventral pallidum, attenuated heroin self-administration (Caille & Parsons, 2006). Together, these results suggested that rimonabant exerts its effects on opioid self-administration independent of dopaminergic activity.

Another prospective utility for CB1 antagonists is for the treatment of alcoholism. A litany of preclinical data has demonstrated rimonabant's efficacy in numerous assays of alcohol-related behavior (for review, see Colombo et al., 2007). A pioneering study from George Kunos' laboratory revealed many key findings regarding interactions between the endocannabinoid system, age and ethanol intake (Wang, Liu, Harvey-White, Zimmer, & Kunos, 2003). Pharmacologic or genetic inhibition (i.e., CB1 -/-) of CB1 receptor activity was shown to reduce

ethanol preference in a two-bottle choice paradigm in younger (6-10 week old) wild-type C57BL/6 mice (Wang et al., 2003). Alcohol preference in mature (26-48 week old) mice was reduced compared to younger mice and rimonabant had no effect on ethanol intake or preference in either genotype at this age range (Wang et al., 2003). No significant age-related differences in CB1 receptor density were noted in a variety of brain regions, including cerebellum, limbic forebrain, amygdala and hypothalamus. However, agonist-stimulated binding was decreased in the limbic forebrain samples (containing nucleus accumbens and anterior cingulate cortex) of old mice relative to young mice, suggesting that the age-related decline in ethanol preference might coincide with decreases in CB1 receptor signaling (Wang et al., 2003).

A clinical study of rimonabant's efficacy in individuals meeting DSM-IV criteria for alcohol dependence conducted in Northern Europe found a non-significant trend of rimonabant (20 mg/day) to increase time to first drink and first heavy drinking period (4 drinks in females, 6 in males) (Soyka et al., 2008). The authors suggested the short treatment period and large percentage of placebo patients who failed to relapse (40.3%) as factors that might have contributed to the observed lack of efficacy of rimonabant. Attrition rates were similar across treatment groups, but discontinuation rates due to treatment-emergent adverse events were higher in patients receiving placebo (7.9%) than rimonabant (4.6%) (Soyka et al., 2008). Additionally, 3 suicide attempts were reported in placebo patients compared to 1 in the experimental group (groups contained a near-identical number of subjects). Although rimonabant failed to significantly improve the primary outcome measures in this report, the relatively low incidence of adverse effects, especially suicide attempts, is an equally important finding.

Appetite Regulation

Perhaps the most promising application for rimonabant was for treatment of obesity. Preclinical data indicated rimonabant decreased food consumption and body weight in free feeding rats, but did not alter water intake (Colombo et al., 1998). Rimonabant also diminished food-maintained operant responding and free feeding access across a variety of diets (high fat, high carbohydrate, standard chow) in rats (high fat, high carbohydrate, standard chow; McLaughlin et al., 2003). In mice, genetic deletion of CB1 receptors resulted in lower progressive ratio breakpoints in operant responding maintained by a sweet reinforcer (Ensure) compared to wild-type mice (Ward & Dykstra, 2005). This enhanced preference for a sweet reward in wild-type mice was abolished by rimonabant challenge (Ward & Dykstra, 2005). No differences in breakpoints maintained by fat (corn oil) reinforcer were noted between genotypes. In fasted mice, rimonabant dose-dependently decreased chow consumption without disrupting locomotor activity and reversed THC-induced increases in food consumption (Wiley et al., 2005). Foltin and Haney (2007) reported that rimonabant and amphetamine decreased intake of both standard food pellets and pieces of candy in non-human primates. Amphetamine also increased the latency to obtain the first food pellet, but not candy, whereas rimonabant did not alter the latency to obtain either reinforcer (Foltin & Haney, 2007). Hence, differential effects of rimonabant on food type have been noted by some (Ward & Dykstra, 2005), but not others (McLaughlin et al., 2003; Foltin & Haney, 2007). These discrepancies may be attributable to any number of differences between studies, such as species, reinforcement schedule, and consistency of reinforcer (liquid vs. solid). Collectively, these and other preclinical findings demonstrating rimonabant's efficacy in attenuating feeding behavior necessitated research in the clinical domain.

Sanofi-Aventis proceeded with a series of rimonabant in obesity (RIO) clinical trials to evaluate rimonabant's efficacy and safety as an anti-obesity agent. These trials were 1 or 2 years in duration with primary endpoints being weight loss and weight maintenance. A meta-analysis of the RIO studies found that patients receiving 20 mg/day rimonabant lost significantly more weight than the placebo group (Christensen, Kristensen, Bartels, Bliddal, & Astrup, 2007). This effect was consistent across all 4 trials, with those receiving rimonabant being 5 times more likely to lose $\geq$ 10% of their body weight (Christensen et al., 2007). Baseline and subsequent quarterly assessments of mood were collected using the hospital and anxiety depression scale (HADS). A significant increase in anxiety, but not depression, score was noted for the experimental group (Christensen et al., 2007). However, assessment of total adverse events revealed a higher incidence of adverse events in rimonabant patients (86.0% compared to 81.8% in placebo group; Christensen et al., 2007). Further analysis of these data revealed significantly higher attrition rates in rimonabant patients attributed to depressed mood disorders and anxiety (Christensen et al., 2007).

As part of its meta-analysis efforts, the U.S. Food and Drug Administration (FDA) commissioned an independent review of source documentation, whereby suicidality events were inferred from patient narratives. A total 66 cases were flagged as possible or definite cases of suicidality between placebo (n=20) and rimonabant patients (46). The majority of cases were classified as suicidal ideation, with rimonabant patients 3 times more likely to experience suicidal ideation than placebo (n=39 vs. 13, respectively). These findings ultimately led to rimonabant being withdrawn from the market in the European Union in early 2009. However, only 4 cases of attempted suicide were noted in the rimonabant group, relative to 7 attempts in the placebo group. Moreover, a significant number of patients seeking treatment for obesity also

experience depression (Wadden et al., 2001), making this a particularly vulnerable population for adverse psychiatric events.

The case for rimonabant and further exploration of CB1 receptor antagonists

Rimonabant has clinically validated efficacy in combating the three leading preventable causes of death in the U.S.: obesity, tobacco and alcohol-related illnesses (Mokdad, Marks, Stroup, & Gerberding, 2004). These global threats to health manifest themselves in myriad well-reported medical consequences, such as heart disease, type 2 diabetes, lung cancer, liver cirrhosis, and deaths attributed to alcohol overdose or automobile accidents. The therapeutic potential of CB1 antagonists previously described in these and other areas provide a compelling argument to continue to pursue a target in these areas. A neutral antagonist, for example, might be predicted to lack the psychiatric-related issues reported for rimonabant in clinical trials. Although drug manufacturers are understandably reluctant to pursue such efforts following the withdrawal of rimonabant from the market, the hypothetical benefits of a CB1 antagonist in a number of therapeutic situations warrant further research to this end.

Pharmacological profile of rimonabant analogs

Following the successful development of the CB1 antagonist rimonabant and subsequent characterization of its pharmacological profile, attempts to create rimonabant analogs were initiated. Indeed, evaluating the structure-activity relationship (i.e., evaluating how manipulations to a compound's chemical structure alter its behavioral and neurochemical effects) of rimonabant can provide valuable information pertinent to the actions of the parent compound and also lead to the discovery of more specific and/or potent CB1 antagonists. Thus, development and testing of rimonabant analogs serves as an avenue towards further understanding cannabinoid pharmacology and possible discovery of new preclinical tools with

potential therapeutic applications. Several structure-activity relationship reports on rimonabant analogs retaining the central pyrazole core of the parent compound (see Figure 1) have provided insight into their neurochemical and behavioral activity.

Two of these reports focused on a series of pyrazole rimonabant analogs (synthesized by Organix Inc.) that investigated their binding affinity at CB1 receptors, and ability to elicit or attenuate cannabimimetic effects in the mouse tetrad. Drugs that produce hypothermia, catalepsy, antinociception and inhibit spontaneous activity (tests that comprise the tetrad) at similar doses are likely to possess cannabinergic activity (Martin et al., 1991). These effects are reversed by the CB1 antagonist, rimonabant (Compton et al., 1996), but not by the CB2 antagonist SR144528 (Wiley et al., 2002). Furthermore, agonist potency for producing these effects is correlated with CB1 binding affinity in the brain (Compton et al., 1993; Adams et al., 1995; Wiley et al., 1998). These findings, in conjunction with the high distribution of CB1 receptors throughout the central nervous system relative to peripherally located CB1 receptors (Pertwee, 1997), strongly suggest that activity of cannabinoid agonists in the tetrad is mediated via central CB1 receptors. Thus, the tetrad has served as a very useful tool in evaluating structure-activity relationships (SAR) of different cannabinoids and understanding requirements for CB1 receptor recognition and activation.

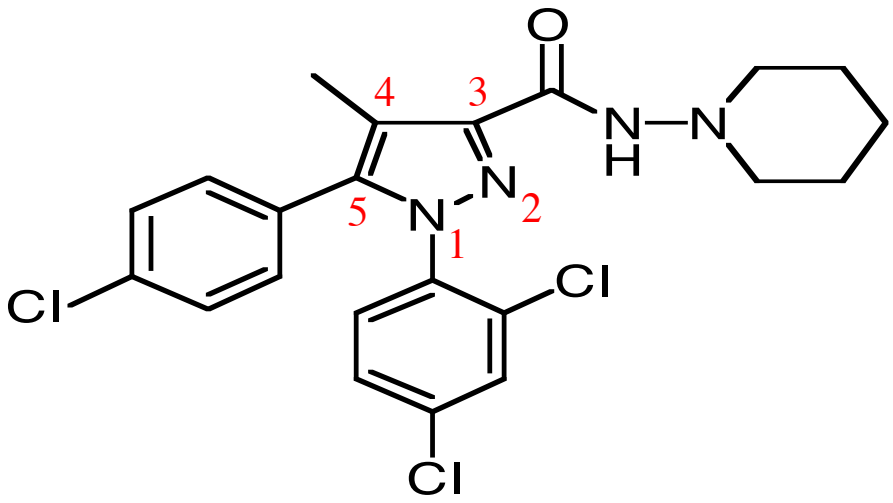


Figure 1. Chemical structure of rimonabant. Points of attachment for substituents of the pyrazole core are denoted in red.

A series of four compounds synthesized by Organix Inc. with 1-substitutent replacements functioned as antagonists or inverse agonists in [^{35}S]GTPγS binding assays (Bass et al., 2002). Not surprisingly, none of these compounds produced tetrad effects on their own; however, only the compound with the highest affinity for CB1 receptors from this series, O-1253, antagonized THC's effects in the tetrad (Wiley et al., 2001). While a low dose (1 mg/kg) of O-1253 blocked THC-induced hypolocomotion, challenge tests with a higher dose (10 mg/kg) stimulated activity (Wiley et al., 2001). When given alone, O-1253 failed to alter locomotor activity (Wiley et al., 2001; Bass et al., 2002). Howlett et al. (2000) reported on two compounds featuring an azido or isothiocyanato substitution in the 1-position that functioned as CB1 receptor antagonists in [^{35}S]GTPγS binding, but at concentrations higher than expected based on their affinity. Interestingly, these compounds also inhibited adenylyl cyclase activity in neuroblastoma cell membranes (Howlett et al., 2000), an effect typically produced by cannabinoid agonists (Howlett & Fleming, 1984). Another investigation of 1-substituent analogs studied revealed that a 2,4-dicholorophenyl substitution produced optimal CB1 receptor affinity relative to other replacements, and functioned as a competitive antagonist in several in vitro assays (Lan et al., 1999). When evaluated together, rimonabant analogs with 1-substituent manipulations retain the antagonist/inverse agonist activity of the parent compound in most in vitro assays, but their ability to reverse THC's tetrad effects was often precluded by poor CB1 receptor affinity.

Work from Wiley et al. (2001) revealed that rimonabant analogs with replacements in the 5- and/or 4-substituent maintained good affinity for the CB1 receptor and readily blocked THC's tetrad effects. One noted exception to this was O-1559, which featured an alkyl group in position 5 in place of the phenyl ring in position 5 and possessed markedly lower CB1 receptor affinity,

providing evidence that the phenyl ring in position 5 is heavily implicated in CB1 receptor recognition (Wiley et al., 2001). Only one compound from this series, O-1710, produced inverse agonist activity in [^{35}S]GTPγS binding assays, but decreased locomotor activity during the first hour compared to the vehicle treated cohort (Bass et al., 2002).

Another interesting compound from this series was O-1691, which featured an alkyl group attached to the phenyl ring in position 5 and replaced the 4-methyl group in position 4 with a bromine. This compound possessed strong CB1 receptor affinity (as did others with an alkyl group attached to the phenyl ring in position 5), stimulated locomotor activity alone, and functioned as an antagonist in [^{35}S]GTPγS binding (Bass et al., 2002). Moreover, O1691 reversed THC-induced antinociception and hypothermia, but failed to ameliorate THC-induced hypolocomotion (Wiley et al., 2001). One potential interpretation of these findings is that the locomotor effects of rimonabant and its analogs occurs independently of CB1 receptor binding, as no significant correlation was found between CB1 affinity and locomotor counts (Bass et al., 2002). Alternatively, these seemingly discrepant locomotor findings may also be attributable to variations in the locomotor protocols used. Bass et al. (2002) evaluated locomotor activity across 2 h, compared to a 10 min session utilized in the tetrad studies (Wiley et al., 2001). Another series of 5-substituent analogs synthesized revealed that a *para*-substitution produced enhanced CB1 receptor affinity and selectivity relative to compounds with *ortho*-substitutions (Lan et al., 1999).

Results from molecular modeling suggest possible overlap between the *para*-position of the 5-substituent of rimonabant and the pentyl side chain of THC (Thomas, Gilliam, Burch, Roche, & Seltzman, 1998). SAR investigations of this pentyl side chain have revealed its importance in CB1 receptor affinity (Compton et al., 1993; Martin et al., 1999). In the same vein,

SAR studies with rimonabant analogs also have demonstrated the importance of the phenyl group in the 5-substituent as being a critical determinant of CB1 receptor affinity. For example, replacement of the phenyl group with an alkyl group decreased CB1 receptor affinity by nearly two orders of magnitude compared to an otherwise identical compound retaining the phenyl group (O-1559 vs. O-1690; Bass et al., 2002). In sum, changes in the 1-, or 5- (with or without concomitant changes in position 4) substituent can influence CB1 binding affinity, and consequently, behavioral activity. Such compounds also retain antagonist or inverse agonist activity based on in vitro screens. Thus, these positions on the pyrazole core of rimonabant appear to be involved in CB1 receptor recognition and antagonism.

Perhaps the most interesting finding from investigations of these pyrazole compounds is that certain alterations in position 3 could elicit agonist or partial agonist-like effects in mice (Wiley et al., 2001). Specifically, some compounds with carbon chain 3-substituent replacements, such as O-1269 and O-1270, produced significant decreases in locomotor activity (Bass et al., 2002) and body temperature and increased tail flick latencies (Wiley et al., 2001). While only a few compounds from this series elicited cannabimimetic activity, subsequent SAR investigations of other 3-substituent rimonabant analogs have revealed a number of other compounds with similar activity. For example, O-4332, O-6629 and O-6658 have varying degrees of CB1 receptor affinity and exhibit agonist-like activity in the tetrad (see Table 1).

O-6629 (5-Bromomethyl-2-[5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl]-4,5-dihydrooxazole) and O-6658 (5-Azidomethyl-2-[5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl]-4,5-dihydrooxazole) share similar chemical structures, featuring a bromine or azido substitution in place of the amide group in position 3, respectively. Results from displacement experiments with radiolabeled CP55,940 and

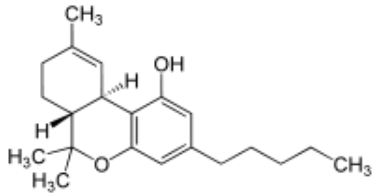
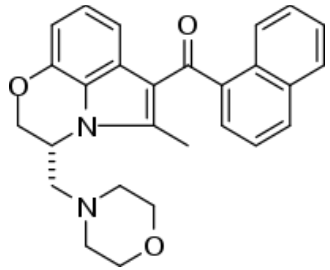
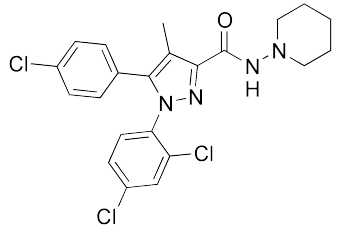
rimonabant demonstrate an approximate 2-fold enhanced CB1 receptor affinity for O-6629 compared to O-6658. Based on their relative CB1 receptor affinity, it was initially speculated that these 3-substituent manipulations conferred cannabinoid agonist properties. However, the inability of rimonabant to reverse the tetrad effects elicited by such compounds did not support that conclusion (JL Wiley, unpublished data).

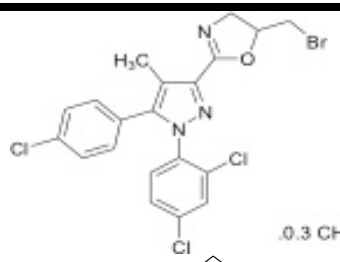
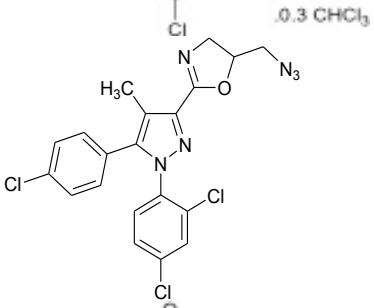
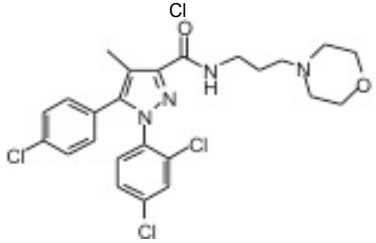
While the tetrad is a quick and efficient preclinical screen for assessing potential cannabinoid agonist activity, other false positives have been noted. Many drug classes elicit one or more effects evaluated in the tetrad (e.g., opioid-induced antinociception) and several antipsychotic drugs (used primarily to treat schizophrenia) produce activity in all four assays (Wiley & Martin, 2003). For example, chlorpromazine, a first-generation antipsychotic, generated tetrad activity with similar potencies noted across all tests. Rimonabant did not reverse antipsychotic-induced tetrad activity, demonstrating that, while non-cannabinoid agents can appear as false positives in this model (Wiley & Martin, 2003), their effects are not blocked by a CB1 antagonist. Thus, the tetrad is arguably more accurate in ruling out, rather than demonstrating, cannabinoid activity (Wiley & Martin, 2003).

Like chlorpromazine, the behavioral activity produced by 3-substituent pyrazole analogs such as O-6629 is not attenuated by a range of rimonabant doses (1-10 mg/kg), Further, these behavioral effects are observed in CB1 knockout mice, suggesting that these compounds are acting independently of established cannabinoid mechanisms. Given the numerous complex interactions between endocannabinoid signaling and other neurochemical targets, the latter hypothesis is certainly reasonable. Combined with the scarcity of published information pertaining to 3-substituent rimonabant analogs and lack of in vitro data suggesting additional sites of action, evaluating these compounds in drug discrimination will serve as an initial avenue

towards investigating potential mechanism/s for their behavioral activity. The paradoxical nature of these compounds (i.e., rimonabant analogs that produce THC-like effects *in vivo* not mediated via established cannabinoid mechanisms) provides an interesting challenge in better understanding the unique pharmacology of these compounds. Therefore, this study is intended to help illuminate the unexpected behavioral effects and mechanism/s of action produced by these rimonabant analogs with 3-substituent substitutions.

Table 1. *Pharmacological profiles of selected cannabinoid agents and pyrazole-derived rimonabant analogs*

Drug	Chemical Structure	CB1 Affinity (K_i)[$]	CB1 Pharmacodynamics	Tetrad activity
THC		37.0[a]	Partial agonist	Yes
WIN 55,212-2		2.5[a]	Full efficacy agonist	Yes
Rimonabant		6.2[a]	Antagonist/ inverse agonist	No, reverses THC tetrad activity

Drug	Chemical Structure	CB1 Affinity (K_i)[$]	CB1 Pharmacodynamics	Tetrad Activity
O-6629 3-substituent		38.4[b]	Unknown[b]	Yes[b]
O-6658 3-substituent		47.5[b]	Unknown[b]	Yes[b]
O-4332 3-substituent		483.8[b]	Unknown[b]	Yes[b]

$ CB1 binding affinity determined by [^{3}H]CP55,940 displacement. All values reported are expressed as nM.

a. Thomas et al., 1998; b. JL Wiley, unpublished data.

Rationale

The endocannabinoid system is involved in numerous physiological processes and engaged in extensive interactions with other neurochemical systems. The development of the selective CB1 receptor antagonist, rimonabant, provided a much-needed pharmacological tool for mechanistic study of the endocannabinoid system and exploration of its complex nature. Given the well-known effects of cannabinoid agonists on appetite, reward and drug abuse-related behavior, a variety of therapeutic applications for CB1 antagonists have been proposed. Preclinical and clinical data have supported this hypothesis, demonstrating rimonabant's efficacy as an anti-obesity pharmacotherapy, as well as a putative treatment for nicotine, opioid and alcohol dependence. The health risks associated with these conditions cannot be understated: Obesity, along with tobacco and alcohol use, are the top three leading preventable causes of death in the United States (Mokdad et al., 2004) and contribute immensely to disease and death globally (Lopez, Mathers, Ezzati, Jamison, & Murray, 2006). Existing treatment options are clearly not sufficient to curb the high number of deaths and illnesses attributed to these factors.

Unfortunately, the emergence of adverse psychiatric events during clinical trials with rimonabant, including increased risk of suicidal ideation, led to manufacturer Sanofi-Aventis withdrawing it from markets where it was available and halting all other clinical studies. Consequently, many other pharmaceutical companies have followed suit and disbanded their CB1 antagonist-focused research efforts. Nevertheless, the wide-reaching therapeutic potential of this treatment strategy necessitates further investigation. For example, the side effect profile of rimonabant may be attributed in part to its inverse agonist activity, whereas a neutral, selective CB1 antagonist might not produce such effects.

Structure-activity relationship studies of rimonabant analogs have provided insight into requisites of CB1 receptor recognition and antagonism. One surprising finding from such investigations was that a number of compounds from a series of pyrazole-derived rimonabant analogs with 3-substituent replacements (e.g., O-6629, O-6658) produced agonist-like effects in the mouse tetrad, a model of cannabimimetic activity. Furthermore, these effects were not reversed by rimonabant challenge, providing evidence that they are not mediated by CB1 receptors. The mechanism/s of action through which these compounds exert their behavioral consequences is not understood. Therefore, the purpose of this study was to characterize the pharmacological properties of these compounds using established behavioral techniques.

The first aim of this study was to replicate tetrad findings with O-6629 and O-6658. Results from these experiments would be used to guide dosing regimens for later studies. It was hypothesized that both O-6629 and O-6658 would produce dose-dependent tetrad activity, with diminished potency relative to intravenous administration.

The second goal was to evaluate these and other pyrazole-derived rimonabant analogs in THC drug discrimination, a preclinical model of the subjective/intoxicating properties of marijuana. The high degree of pharmacological specificity offered by this model makes it an ideal complement to tetrad findings. Selected compounds were evaluated in mice trained to discriminate between the interoceptive stimuli elicited by THC or vehicle to determine whether they engender or modify THC's discriminative stimulus. It was hypothesized that 3-substituent compounds that produced tetrad activity would not produce THC-like discriminative effects or attenuate THC's discriminative stimulus during challenge tests. Results from this series of experiments were expected to reveal similarities or differences between the prototypic

cannabinoid, THC, and rimonabant analogs that elicit a similar cannabimimetic profile, as determined by the tetrad.

The final objective of this project was to train O-6629 as a discriminative stimulus and subsequently investigate potential mechanisms of action. This goal was achieved through three different approaches: First, rimonabant was evaluated for substitution for O-6629's discriminative stimulus. Additionally, O-6629's discriminative stimulus was compared to O-6658, a structurally similar 3-substituent rimonabant analog to determine whether these compounds share similar discriminative stimuli, which would be indicative of shared mechanism of action. The second approach investigated whether a spectrum of cannabinoid agonists share discriminative stimulus effects with O-6629. Test compounds included the phytocannabinoid THC, the potent synthetic agonist WIN 55,212-2, and the endogenous cannabinoid anandamide. Finally, representative compounds from a number of drug classes with noted endocannabinoid system interactions were evaluated to determine whether they engender O-6629-like discriminative stimulus effects. Test compounds included cocaine, diazepam, morphine and nicotine.

It was predicted that O-6629 would share discriminative stimulus effects with other 3-substituent pyrazole analogs that display similar behavioral profiles in the tetrad, but would not generalize to other cannabinoids. Given the extensive nature of endocannabinoid signaling with other neurochemical systems, it was difficult to predict which, if any, of the proposed test compounds would produce O-6629 discriminative stimulus effects. The potential for negative data notwithstanding, this initial exploration into the pharmacological properties of O-6629 and other 3-substituent pyrazole rimonabant analogs, at minimum, ruled out involvement from a number of major receptor classes, including dopamine, μ-opioid, GABA and nicotinic

acetylcholine systems. Further, should O-6629 serve as a discriminative stimulus in the absence of other external manipulations (e.g., chronic cannabinoid treatment or taste aversion), this would differentiate this analog from its parent compound.

In sum, despite an elevated risk of adverse psychiatric effects reported from clinical investigations of rimonabant, the impetus to develop better pharmacotherapies for treatment of obesity and various substance abuse disorders warrants further evaluation of CB1 receptor antagonists. Such examinations have revealed a series of rimonabant analogs with 3-substituent replacements that possess an uncharacteristic behavioral profile given their chemical derivation. Therefore, this project sought to explore potential mechanisms of action for this class of compounds utilizing drug discrimination, a behavioral assay that possesses an exceptionally high degree of pharmacological specificity. This initial determination provided insight into the behavioral activity of 3-substituent pyrazole analogs and excluded potential sites of their actions.

Methods

Experiment 1—Cannabimimetic effects of 3-substituent rimonabant analogs

Subjects

Forty-eight adult male, experimentally naïve C57BL/6J mice (20-25g) obtained from Jackson Laboratories (Bar Harbor, ME) served as subjects. Mice were group housed (4-5/group) in clear plastic cages with fitted tops and corncob bedding and had unlimited access to food (Teklad chow; Harlan, Indianapolis, IN) and water in the home cage. Mice were housed in a light (12 hour light-dark cycle, lights on at 0600) and temperature (22-24° C) controlled vivarium. The evening before testing, mice were randomly assigned and sorted into one of eight treatment conditions (n=6 per treatment condition; 0, 1, 3, 10 mg/kg O-6629 or O-6658) and were

transported to the laboratory where experimental test sessions would occur to allow them to adjust to ambient room temperatures. The *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy Press, 1996) was followed and the Institutional Animal Care and Use Committee at Virginia Commonwealth University approved the procedures described for all experiments described herein.

Drugs

O-6629 and O-6658 (Organix Inc., Woburn, MI) were dissolved in a vehicle consisting of ethanol, Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ), and saline at a ratio of 1:1:18. Drugs were administered subcutaneously (s.c.) 60 min prior to the start of the experiments at a volume of 10 ml/kg body weight.

Apparatus

Assessment of spontaneous activity in mice occurred in standard activity chambers containing 8 photocell beams enclosed in sound and light attenuating cubicles (Med-Associates, St. Albans, VT). Body temperature was measured by a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH) and a thermistor probe inserted 25 mm into the rectum. Antinociception was assessed using a standard tail flick apparatus. Catalepsy was evaluated using a modified version of the ring immobility test (Pertwee, 1972), which was comprised of a metal ring (5.5 cm in diameter) attached to a stand 16 cm above the surface where it was situated.

Procedures

Each mouse was tested in all of the tetrad assays: locomotor activity, tail flick, rectal temperature and ring immobility. Prior to drug administration, rectal temperature and baseline tail flick latency were determined in the mice. The latter procedure involved placing the mouse's

tail on an ambient heat source (i.e., bright light) and latency (in s) for tail removal served as the dependent variable. Typical control latencies were 2-4 s. A 10 s maximal latency was used in order to avoid damage to the mouse's tail. After measurement of temperature and baseline tail flick latency, mice were injected with vehicle or drug. One h later they were placed into individual activity chambers for 10 min. Spontaneous activity was measured as the total number of beam interruptions during the entire session, which was expressed as percent inhibition of the control (vehicle) group's activity. Immediately thereafter, mice were re-tested in the tail flick procedure. Antinociception was expressed as the percent maximum possible effect (%MPE) using a 10 s maximum test latency. %MPE was calculated using the following formula: [(test latency-baseline latency)/(10-baseline latency)*100]. Rectal temperature was re-assessed next and was expressed as the difference between pre- and post-injection rectal temperatures. Next, mice were placed on the ring apparatus, and the amount of time the animals remained cataleptic (i.e., motionless except for respiration; whisker movements were scored as movement) during a 5-min period was recorded. Percent immobility was expressed as [(time immobile/300)*100], whereby 300 represented the total session time (300 s) and the dividend was multiplied by 100 to create a percentage score.

Data analysis

Data for each assay and drug condition were analyzed using a one-way analysis of variance (ANOVA). Significant differences between means were followed by a Dunnett's post-hoc test to identify differences relative to vehicle controls ($p < 0.05$).

Experiment 2—Discriminative stimulus effects of THC: evaluation of 3-substituent rimonabant analogs

Subjects

Sixteen adult male C57BL/6J wild type mice (20-25g) obtained from Jackson Laboratories (Bar Harbor, ME) served as subjects. THC generalization data from a subset of these subjects were previously reported by Wiley et al. (2011). Mice were housed individually in clear plastic cages with fitted tops and corncob bedding. Mice were transported daily (Monday-Friday) from a light (12 hour light-dark cycle, lights on at 0600) and temperature (22-24°C) controlled vivarium to the laboratory for experimental training and testing sessions. After one week of acclimation, subjects were food restricted to 85-90% of their free feeding body weights to initiate the nose poke response and weights were maintained by rationing daily food intake. Water was available *ad libitum* in the home cages. When stable rates of responding were established on both nose poke apertures, subjects were allowed to gradually gain weight as drug discrimination training progressed, provided the mouse maintained 80% accuracy on the appropriate nose poke aperture.

Drugs

THC and rimonabant were obtained from the National Institute on Drug Abuse (Rockville, MD) and dissolved in a mixture of 0.78% Tween-80 (Fischer Scientific, Pittsburgh, PA) and 99.22% saline. O-4332, O-6629 and O-6658 (Organix Inc., Woburn, MI) were dissolved in a vehicle consisting of ethanol, Emulphor-620 (Rhone-Poulenc, Inc., Princeton, NJ), and saline at a ratio of 1:1:18. All compounds were administered s.c. at a volume of 10 ml/kg body weight. THC and its vehicle were administered 30 min pre-session, rimonabant was administered 40 min pre-session and rimonabant analogs (i.e., O-4332, O-6629, O-6658) were administered 60 min pre-session.

Apparatus

Testing was conducted in eight standard computer-interfaced operant conditioning chambers (Med Associates Inc., Georgia, VT) with two nose poke apertures in the left and right positions (8

cm apart) on the front panel. Each aperture contained an infrared beam that was interrupted when a mouse inserted their snout, which would count as one response. Centered between the apertures was a recessed food receptacle connected to a food hopper that delivered reinforcement (14 mg sweetened pellets; Bio-Serv, Frenchtown, NJ). The inner test chambers consisted of a 15 cm L X 11.5 cm D X 17.5 cm H area surrounded by an aluminum chassis box with a single Plexiglas side door. Test chambers were housed in sound attenuated chambers and ventilation fans provided masking noise. MED-PC software (Med Associates) controlled session parameters and recorded data.

Training procedures

Nose poke training was initiated through an overnight session, during which mice were placed in the operant chamber and trained to insert their snout into either nose poke aperture for a sweetened food pellet on a fixed ratio one (FR1) schedule of reinforcement, in which a pellet was delivered after every nose poke. Following this overnight session, the number of responses on both the right and left apertures was calculated, and the preferred side (the side with the highest number of responses) was determined. Mice were then trained to nose poke on the preferred aperture as the value of the FR requirement was gradually increased over the next several sessions until subjects responded readily under FR10 conditions. Once subjects maintained responding on the preferred side, they were required to follow the same progression (i.e., FR1-FR10) on the non-preferred side. Once stable rates of responding were achieved on both apertures, drug discrimination training commenced.

Drug Discrimination Training

Mice were trained to discriminate 5.6 mg/kg THC vs. vehicle. On days when drug was administered, only responses on the drug-associated aperture were reinforced. On days when

vehicle was administered, only responding on the vehicle-associated aperture was reinforced. Responses on the incorrect aperture reset the ratio requirement on the correct aperture. The position of the drug-associated aperture (left or right) was randomly assigned for all subjects. A double alternation schedule (i.e., DDVVDDVV) was used during training and throughout the remainder of the study.

Drug Discrimination Criteria

Successful acquisition of THC's discriminative stimulus was demonstrated when subjects met the following three criteria for 7 out of 8 consecutive sessions: (1) the first completed FR10 was on the appropriate aperture, (2) 80% or greater of the total responding occurred on the appropriate aperture and (3) response rate equaled or exceeded 0.17 responses per second (10 responses/min). Control tests with vehicle and 5.6 mg/kg THC were administered and passed prior to generalization testing with all new test drugs. During control and test sessions, responses on both apertures were reinforced according to the FR10 schedule and the FR counter was reset if an animal interrupted their responding on one aperture to respond on the opposite one. The three training criteria also had to be met during the most recent training sessions with the training drug and vehicle immediately prior to all test sessions.

Testing procedures

Generalization or substitution testing typically occurred on Tuesdays and Fridays with a minimum of 2 days between tests, provided subjects passed training criteria during their most recent drug and vehicle training sessions. After successful completion of vehicle and THC control tests, a generalization dose effect curve was determined for THC (1-30 mg/kg) in all subjects. Next, doses of rimonabant were tested in combination with the training dose to assess for CB1 mediation of THC's discriminative stimulus. Next, O-4332, O-6629 and O-6658 were tested alone to determine

whether they occasioned THC-appropriate responding or altered THC's discriminative stimulus. In the event that a compound substituted for THC or altered THC's discriminative stimulus, a challenge with rimonabant was conducted. Finally, O-6629 and O-6658 were evaluated in combination with the training dose of THC to determine whether they modified THC's discriminative stimulus. New control tests were performed between each new test drug with the training drug and vehicle to assess THC discriminative stimulus control.

The rate suppressant effects of O-6629 precluded assessments of higher doses more relevant to the dose of O-6629 trained as a discriminative stimulus in experiment 3 (5.6 mg/kg). Thus, following a 1 week washout from their most recent test session (5 weeks from most recent test with O-6658, a 3-substitutent pyrazole compound), mice were treated with 5.6 mg/kg O-6629 once daily for consecutive 15 days to assess for tolerance to its rate suppressant effects. Tolerance to O-6629 was assessed during test sessions with 5.6 mg/kg O-6629 on days 1 and 12, whereby day 1 provided a baseline measure of 5.6 mg/kg O-6629's rate suppressant effects. Then, 5.6 mg/kg O-6629 was tested in combination with 5.6 mg/kg THC on day 15. On training days, subjects were administered 5.6 mg/kg O-6629 immediately following their operant session. Control points with vehicle and THC were conducted during this chronic dosing regimen on days 5 and 8, respectively, to ensure subjects were accurately discriminating between the two conditions amidst post-session injections of O-6629.

Data Analysis

The first FR (FFR) was collected for each test session and served as an index of accurate discrimination during control tests. The number of responses on each aperture was recorded and converted into percent drug lever responding (%DLR) by dividing the number of responses on the drug aperture by total responses on both apertures and multiplying by 100. Responses per second

(RPS) for each session were calculated. ED_{50} values (with 95% confidence intervals, C.I.) were calculated for %DLR data using the least squares method of linear regression with the linear portion of the dose effect curve (Bliss, 1967). ED_{50} values were calculated for test drugs that fully substituted for the training dose of THC (full substitution $\geq$ 80% DLR; partial substitution was $\geq$ 60 to < 80% DLR). A repeated-measures analysis of variance (ANOVA) comparing responses per minute was performed for each drug (GraphPad Prism 5 for Mac OS X software; GraphPad Software Inc., La Jolla, CA). Significant ANOVAs were followed by Dunnett's post-hoc tests ($p < 0.05$ compared to vehicle). Subjects that failed to make ten or more responses during the course of the test session had their %DLR data for that data point excluded from analysis.

Experiment 3—Discriminative stimulus effects of O-6629

Subjects

Thirty-one adult male C57BL/6J mice (Jackson Laboratories) served as subjects. All other details same as experiment 2.

Drugs

Chlorpromazine hydrochloride and diazepam obtained from Sigma-Aldrich (St. Louis, MO), and cocaine hydrochloride, morphine sulfate, and nicotine hydrogen tartrate salt were obtained from the National Institute on Drug Abuse and dissolved in physiological saline. WIN 55,212-2 was obtained from Sigma-Aldrich and dissolved in a vehicle consisting of ethanol, Emulphor-620, and saline at a ratio of 1:1:18. Anandamide was obtained from Organix Inc. and dissolved in 0.78% Tween-80 and 99.22% saline. Cocaine and diazepam were administered i.p. 10 and 15 min pre-session, respectively. Morphine was administered s.c. 20 min pre-session.

Anandamide, chlorpromazine and WIN 55,212-2 were administered s.c. 30 min pre-session. Details for O-6629, O-6658, THC, and rimonabant are same as experiment 2.

Apparatus

Operant conditioning equipment described in experiment 2 was also used to conduct drug discrimination sessions for experiment 3.

Training procedures

Nose poke training proceeded as described in experiment 2.

Drug Discrimination Training

Based upon test results obtained with O-6629 in experiments 1 and 2, a training dose of 5.6 mg/kg O-6629 was determined. Thus, mice were trained to discriminate 5.6 mg/kg O-6629 vs. vehicle. All other training details (e.g., acquisition criteria, data analysis) were identical to those described in experiment 2.

Testing procedures

Once subjects met acquisition criteria and completed a dose effect curve with O-6629 (1-10 mg/kg), they were randomly assigned to one of three testing groups, each with a specific goal (see Table 2). This three-group design was employed to facilitate data collection and maintain identical drug history within subsets of the animals. The aim of the first group was to investigate the structure-activity relationship among rimonabant analogs. Thus, O-6658 and rimonabant were tested in relation to O-6629's discriminative stimulus. The second group was tested with a variety of cannabinoids, including the plant-derived partial agonist THC, the potent full efficacy agonist WIN 55,212-2, and the endogenous cannabinoid anandamide. Finally, the third group was tested with a variety of prototypical compounds from a number of major drug classes noted for their interactions with the endocannabinoid system, including cocaine, diazepam, morphine,

and nicotine. Additionally, chlorpromazine was tested based on its ability to produce activity in all four assays of the tetrad within a limited dose range (Wiley & Martin, 2003). All test compounds were evaluated alone to determine whether they elicited similar discriminative stimulus effects as O-6629. If a compound substituted for O-6629, an appropriate challenge test was conducted to further evaluate the mechanism/s of said effect. For example, if nicotine attenuated O-6629's discriminative stimulus, mecamylamine would be used to determine whether that attenuation was due to nicotinic cholinergic activity or some other non-specific effect (e.g., postsynaptic dopamine release). Table 2 provides a summary of test conditions for these experiments.

Data Analysis

Drug discrimination data were analyzed as described in experiment 2.

Table 2—Experiment 3 studies

	Goals	Generalization tests
Group 1	SAR of rimonabant and related pyrazole compounds	Rimonabant O-6658
Group 2	Evaluation of cannabinoids	THC Anandamide WIN 55,212-2
Group 3	Evaluating other potential targets responsible for O-6629's discriminative stimulus effects	Cocaine (DA) Morphine (μ-opioid) Nicotine (ACh)

Results

Experiment 1—Cannabimimetic effects of 3-substituent rimonabant analogs

Pharmacological profile of O-6629 in the tetrad

Data from the evaluation of O-6629 in the tetrad battery are presented in Figure 2. O-6629 significantly decreased spontaneous activity, F (3, 20) = 88.38, $p < 0.05$. Post-hoc analysis revealed that activity at the 10 and 30 mg/kg doses was significantly decreased compared to vehicle, $p < 0.05$. O-6629 produced significant antinociception in the tail flick assay, F (3, 20) = 3.26, $p < 0.05$. Post-hoc analysis revealed 30 mg/kg significantly increased %MPE compared to vehicle, $p < 0.05$. O-6629 produced significant hypothermia, F (3, 20) = 78.16, $p < 0.05$. Post-hoc analysis revealed body temperatures following 10 and 30 mg/kg doses were significantly decreased compared to vehicle, $p < 0.05$. O-6629 produced significant catalepsy in the ring immobility task, F (2, 15) = 17.06, $p < 0.05$. Post-hoc analysis revealed significant catalepsy following 10 mg/kg administration, $p < 0.05$. All subjects treated with 30 mg/kg were unable to remain situated on the ring, and after replacing each mouse back on the ring 5 times, assessment of catalepsy ceased. Thus, no data are presented for this dose.

Pharmacological profile of O-6658 in the tetrad

Data from the evaluation of O-6658 in the tetrad battery are presented in Figure 3. O-6658 significantly decreased spontaneous activity, F (3, 20) = 141.30, $p < 0.05$. Post-hoc analysis revealed that activity at the 10 and 30 mg/kg doses was significantly decreased compared to vehicle, $p < 0.05$. A significant antinociceptive effect of O-6658 was not observed, F (3, 20) = 3.06, $p > 0.05$. O-6658 produced significant hypothermia, F (3, 20) = 164.00, $p < 0.05$. Post-hoc analysis revealed body temperatures following 10 and 30 mg/kg doses were significantly decreased compared to vehicle, $p < 0.05$. O-6658 produced significant catalepsy in

the ring immobility task, F (2, 15) = 7.71, $p < 0.05$. Post-hoc analysis revealed significant catalepsy following 10 mg/kg administration. As was the case with O-6629, all subjects treated with 30 mg/kg O-6658 were unable to remain situated on the ring following five attempts to reinitiate assessment of catalepsy. Thus, no data are presented for this dose.

Experiment 2—Discriminative stimulus effects of THC: evaluation of 3-substituent rimonabant analogs

THC's discriminative stimulus: acquisition and generalization

Acquisition data for THC's discriminative stimulus are presented in Figure 4. Thirteen out of 16 subjects met acquisition criteria (correct FFR, $\geq$ 80% condition-appropriate responding, response rate $\geq$ 0.17 responses/s) for 7 out of 8 consecutive sessions, consequently meeting acquisition criteria in a mean of 29.50 sessions (SEM = 2.18). Range to acquisition was 14-49 sessions. Subjects that failed to meet acquisition criteria after 100 training sessions were excluded from the study.

Results from generalization testing with THC in all subjects are presented in Figure 5. Dose-dependent generalization with THC was observed, with a calculated ED50 of 2.37 mg/kg (95% C.I. = 1.81-3.09). Partial substitution occurred at 3 mg/kg and full substitution was seen following administration of 5.6, 10 and 30 mg/kg doses. Analysis of response rate data revealed a significant effect of dose, F (7, 84) = 10.42, $p < 0.05$. Compared to vehicle, response rates were significantly decreased at 10 and 30 mg/kg doses, $p < 0.05$.

Rimonabant challenge

Results from challenge tests with rimonabant against the training dose of THC (5.6 mg/kg) are presented in Figure 6. Rimonabant dose-dependently attenuated THC's

discriminative stimulus, with a calculated AD50 of 0.62 mg/kg (95% C.I. = 0.44 – 0.87). Analysis of response rate data revealed a significant effect of dose, F (5, 25) = 3.46, $p < 0.05$. Compared to vehicle, response rates were significantly decreased during control tests with the highest dose of rimonabant tested (1 mg/kg), $p < 0.05$.

O-4332 generalization

Results from generalization testing with O-4332 are presented in Figure 7. O-4332 failed to substitute for THC, eliciting a maximum 22.29% THC-appropriate responding at the 3 mg/kg dose. Analysis of response rate data revealed a significant effect of dose, F (4, 32) = 16.02, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by 10 and 30 mg/kg doses, $p < 0.05$. Additionally, response rates for THC control tests were significantly higher than vehicle, $p < 0.05$.

O-6629 generalization

Results from generalization testing with O-6629 are presented in Figure 8. O-6629 failed to substitute for THC, eliciting a maximum 11.45% THC-appropriate responding at the 3 mg/kg dose. Analysis of response rate data revealed a significant effect of dose, F (5, 15) = 5.39, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by the 10 mg/kg dose, $p < 0.05$. At this dose, all subjects made fewer than 10 responses during the test session; thus, no generalization data are presented for this dose.

O-6629 challenge

Results from a challenge test with O-6629 against the training dose of THC are presented in Figure 9. The 3 mg/kg dose of O-6629 resulted in a reduction of THC-appropriate responding below full substitution criteria (71.82% DLR); however, inspection of data for individual animals revealed that full substitution was observed in 3 out of 4 mice responding at this dose. Analysis

of response rate data revealed a significant effect of dose, F (4, 28) = 14.91, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by 3.0 mg/kg O-6629 in combination with the THC training dose, $p < 0.05$.

O-6658 generalization

Results from generalization testing with O-6658 are presented in Figure 10. O-6658 failed to substitute for THC, eliciting a maximum 3.95% THC-appropriate responding at the 1 mg/kg dose. O-6658 failed to significantly alter response rates, although a trend towards a decrease in responding was observed, F (4, 20) = 2.57, $p = 0.07$.

O-6658 challenge

Results from a challenge test with O-6658 against the training dose of THC are presented in Figure 11. No dose tested resulted in a reduction of THC-appropriate responding below full substitution criteria. Analysis of response rate data revealed a significant effect of dose, F (4, 28) = 5.90, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by 1.0 and 3.0 mg/kg O-6658 in combination with the THC training dose, $p < 0.05$.

O-6629 tolerance

Results from the chronic dosing experiment assessing tolerance development to the rate suppressant effects of 5.6 mg/kg O-6629 (dose trained as a discriminative stimulus in experiment 3) are presented in Figure 12. Analysis of response rate data revealed a significant effect of time and treatment, F (4, 20) = 5.13, $p < 0.05$. Compared to vehicle, response rates were significantly decreased following initial treatment with O-6629 (day 1), $p < 0.05$. Following 12 days of chronic treatment, response rates did not differ significantly from vehicle, $p > 0.05$. However, during a challenge test with 5.6 mg/kg O-6629 against the training dose of THC, response rates were significantly decreased compared to vehicle, $p < 0.05$. Visual inspection of generalization

data revealed a slight increase in %THC-appropriate responding on day 12 compared to day 1 (19.45% vs. 5.33%, respectively). Evaluation of individual data revealed that full substitution for THC was observed in one subject, as opposed to a general increase in THC-appropriate responding among all subjects. Thus, the chronic dosing regimen did not unmask a meaningful alteration in O-6629's ability to produce THC-like discriminative stimulus effects. Challenge test data revealed a mean of 52.71% THC-appropriate responding. Evaluation of individual data in subjects performing ≥ 10 responses during the challenge test (n=3/6) revealed marked response discrepancies among individual subjects (16.47%, 46.15%, 95.52%).

Experiment 3—Discriminative stimulus effects of O-6629

O-6629's discriminative stimulus: acquisition and generalization

Acquisition data for O-6629's discriminative stimulus are presented in Figure 13. Twenty-seven out of 31 subjects met acquisition criteria for 7 out of 8 consecutive sessions, consequently meeting acquisition criteria in a mean of 69.56 sessions (SEM = 6.13). Range to acquisition was 26-133 sessions. Subjects not meeting acquisition criteria following 150 training sessions were removed from the study. An additional subject passed away prior to completing the O-6629 generalization curve.

Results from generalization testing with O-6629 in all subjects are presented in Figure 14. Dose-dependent generalization with O-6629 was observed, with an ED50 of 2.15 mg/kg (95% C.I. = 1.72-2.68). Full substitution was seen following administration of 5.6 and 10 mg/kg doses. Analysis of response rate data revealed a significant effect of dose, F (5, 125) = 18.02, $p < 0.05$. Compared to vehicle, response rates were significantly decreased at 5.6 and 10 mg/kg doses, $p < 0.05$.

O-6658 generalization

Results from generalization testing with O-6658 are presented in Figure 15. O-6658 fully substituted for O-6629, eliciting a maximum 91.86% O-6629-appropriate responding at the 10 mg/kg dose. Analysis of response rate data revealed a non-significant effect of dose, F (5, 15) = 1.75, $p > 0.05$.

Rimonabant generalization

Results from generalization testing with rimonabant are presented in Figure 16. Rimonabant did not substitute for O-6629, eliciting a maximum 35.59% O-6629-appropriate responding at the 0.1 mg/kg dose. Analysis of response rate data revealed a significant effect of dose, F (6, 24) = 3.53, $p < 0.05$. However, post-hoc analysis did not reveal any significant differences in response rate compared to vehicle.

THC generalization

Results from generalization testing with THC are presented in Figure 17. THC did not substitute for O-6629, eliciting a maximum 51.60% O-6629-appropriate responding at the 1.0 mg/kg dose. Analysis of response rate data revealed a significant effect of dose, F (5, 25) = 3.05, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by the 10 mg/kg dose, $p < 0.05$.

Anandamide generalization

Results from generalization testing with anandamide are presented in Figure 18. Anandamide did not substitute for O-6629, eliciting a maximum 50.03% O-6629 appropriate responding at the 10 mg/kg dose. Analysis of response rate data revealed a non-significant effect of dose, F (5, 15) = 2.60, $p > 0.05$.

WIN 55,212-2 generalization

Results from generalization testing with WIN 55,212-2 are presented in Figure 19. WIN 55,212-2 produced partial substitution for O-6629, eliciting a maximum 72.71% O-6629-appropriate responding at the 1.7 mg/kg dose. Partial substitution was also observed following administration of 3.0 mg/kg WIN 55,212-2. Analysis of response rate data revealed a significant effect of dose, F (6, 18) = 6.97, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by the 3 mg/kg dose, $p < 0.05$.

Morphine generalization

Results from generalization testing with morphine are presented in Figure 20. Morphine did not substitute for O-6629, eliciting a maximum 19.16% O-6629 appropriate responding at the 3 mg/kg dose. Analysis of response rate data revealed a significant effect of dose, F (4, 24) = 26.17, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by 1, 3 and 10 mg/kg doses, $p < 0.05$. All subjects made fewer than 10 responses during the 10 mg/kg test session; thus, no generalization data are presented for this dose. Additionally, response rates for the O-6629 control test were significantly decreased relative to vehicle, $p < 0.05$.

Nicotine generalization

Results from generalization testing with nicotine are presented in Figure 21. Nicotine (1 mg/kg) produced partial substitution for O-6629, eliciting a maximum 76.88% O-6629 appropriate responding. Inspection of individual data revealed full substitution occurred in 3 out of 4 subjects tested at this dose. A higher dose of nicotine (1.7 mg/kg) also generated partial substitution for O-6629. Analysis of response rate data revealed a significant effect of dose, F (5, 15) = 7.49, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by 1.7

mg/kg nicotine, $p < 0.05$. Additionally, response rates for the O-6629 control test were significantly decreased relative to vehicle, $p < 0.05$.

Five subjects were initially included in this dose effect determination, but one subject died before completing the dose effect curve; thus, data from four subjects are presented. It should be noted that this mouse responded almost exclusively on the vehicle-paired nose poke aperture at doses tested (0.1-1 mg/kg), but that partial substitution still would have been observed at the 1 mg/kg dose if their data were included.

Cocaine generalization

Results from generalization testing with cocaine are presented in Figure 22. Cocaine did not substitute for O-6629, eliciting a maximum 49.69% O-6629-appropriate responding at the 1 mg/kg dose. Analysis of response rate data revealed a significant effect of dose, F (7, 28) = 10.95, $p < 0.05$. Compared to vehicle, response rates were significantly decreased by 10 and 30 mg/kg doses, $p < 0.05$. Additionally, response rates for the O-6629 control test were significantly decreased relative to vehicle, $p < 0.05$.

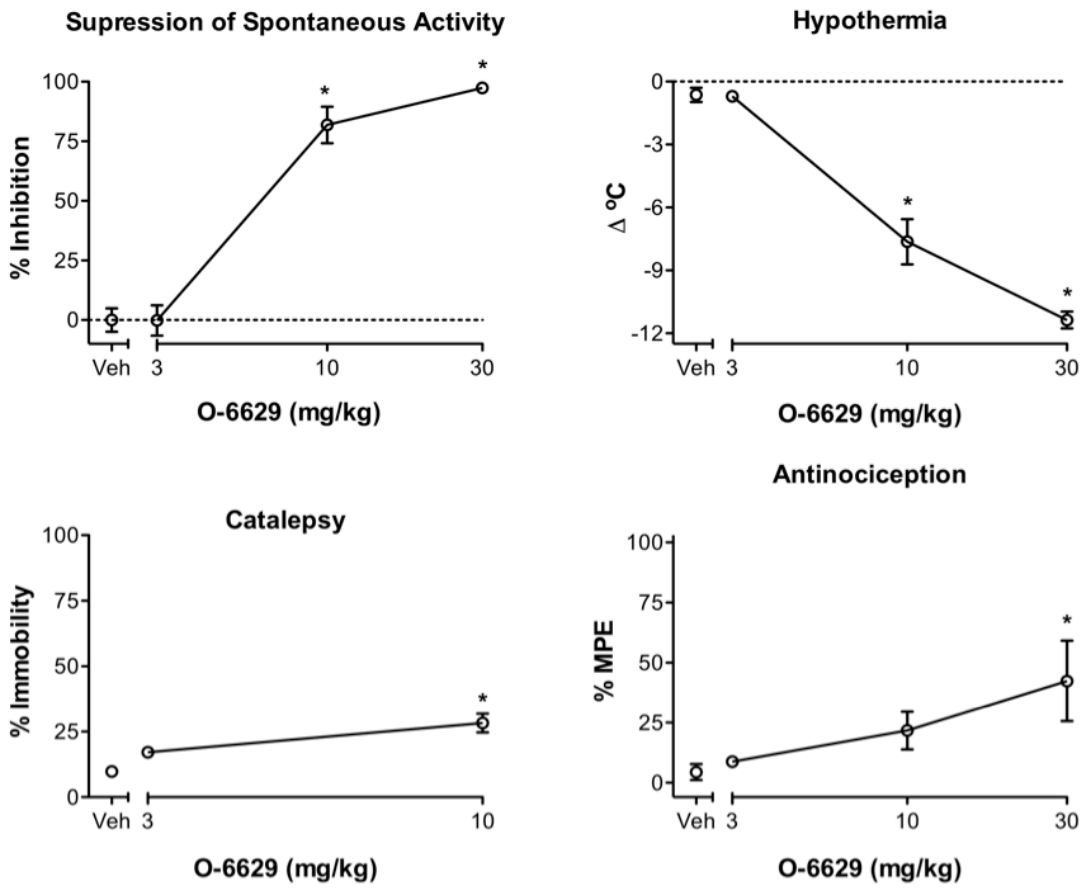


Figure 2. Pharmacological profile of O-6629 in the tetrad. Effects of O-6629 on spontaneous activity (top left panel), body temperature (top right panel), catalepsy (bottom left panel) and tail flick latency (bottom right panel). Values represent mean (±SEM) of 6 mice per treatment condition. Asterisks (*) denote significant difference compared to vehicle, $p < 0.05$.

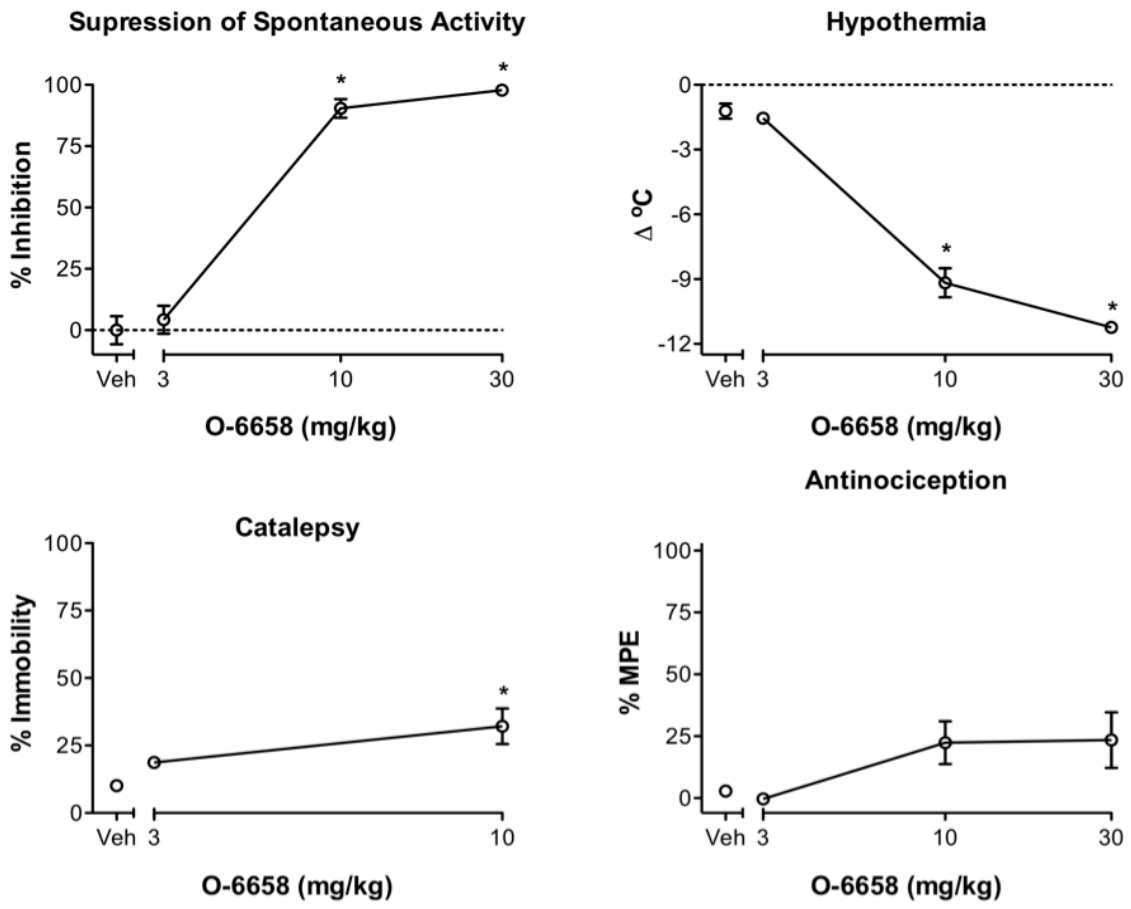


Figure 3. Pharmacological profile of O-6658 in the tetrad. Effects of O-6658 on spontaneous activity (top left panel), body temperature (top right panel), catalepsy (bottom left panel) and tail flick latency (bottom right panel). All other details same as Figure 2.

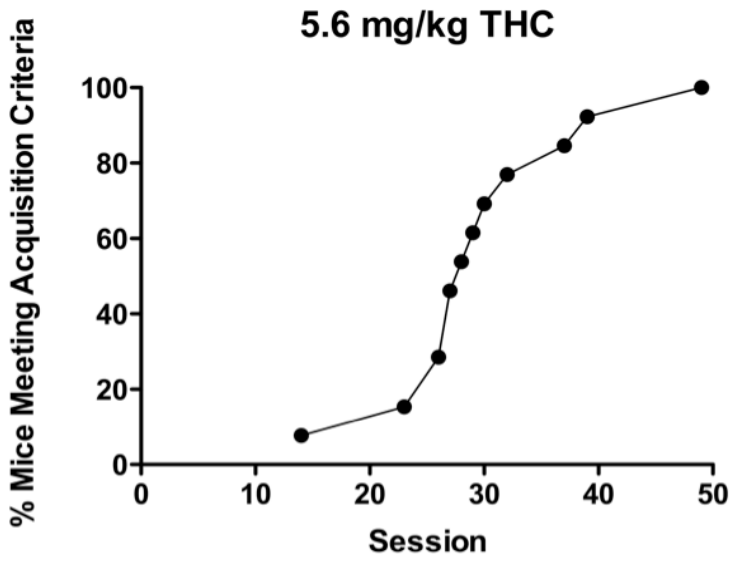


Figure 4. Acquisition of THC's discriminative stimulus. Values represent percentage of subjects meeting acquisition criteria across sessions (N = 13/16).

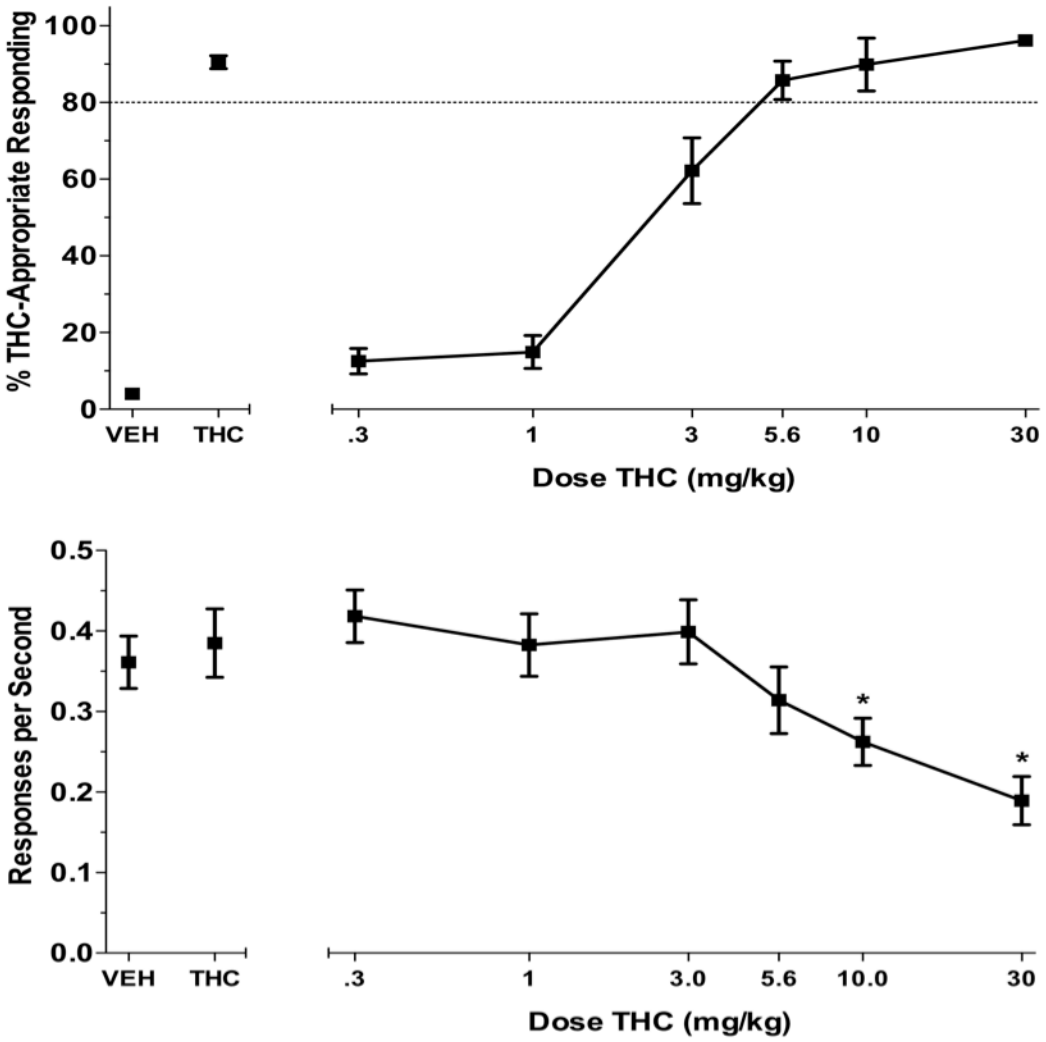


Figure 5. THC generalization. Effects of THC on mean percentage THC-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) in mice trained to discriminate 5.6 mg/kg THC from vehicle (N = 13). Values above VEH and THC represent control test data collected prior to conducting the dose effect determination. The dashed line at 80% drug lever responding indicates full generalization to the training dose. For response rate data, asterisks (*) denote significant differences compared to vehicle control, $p < 0.05$.

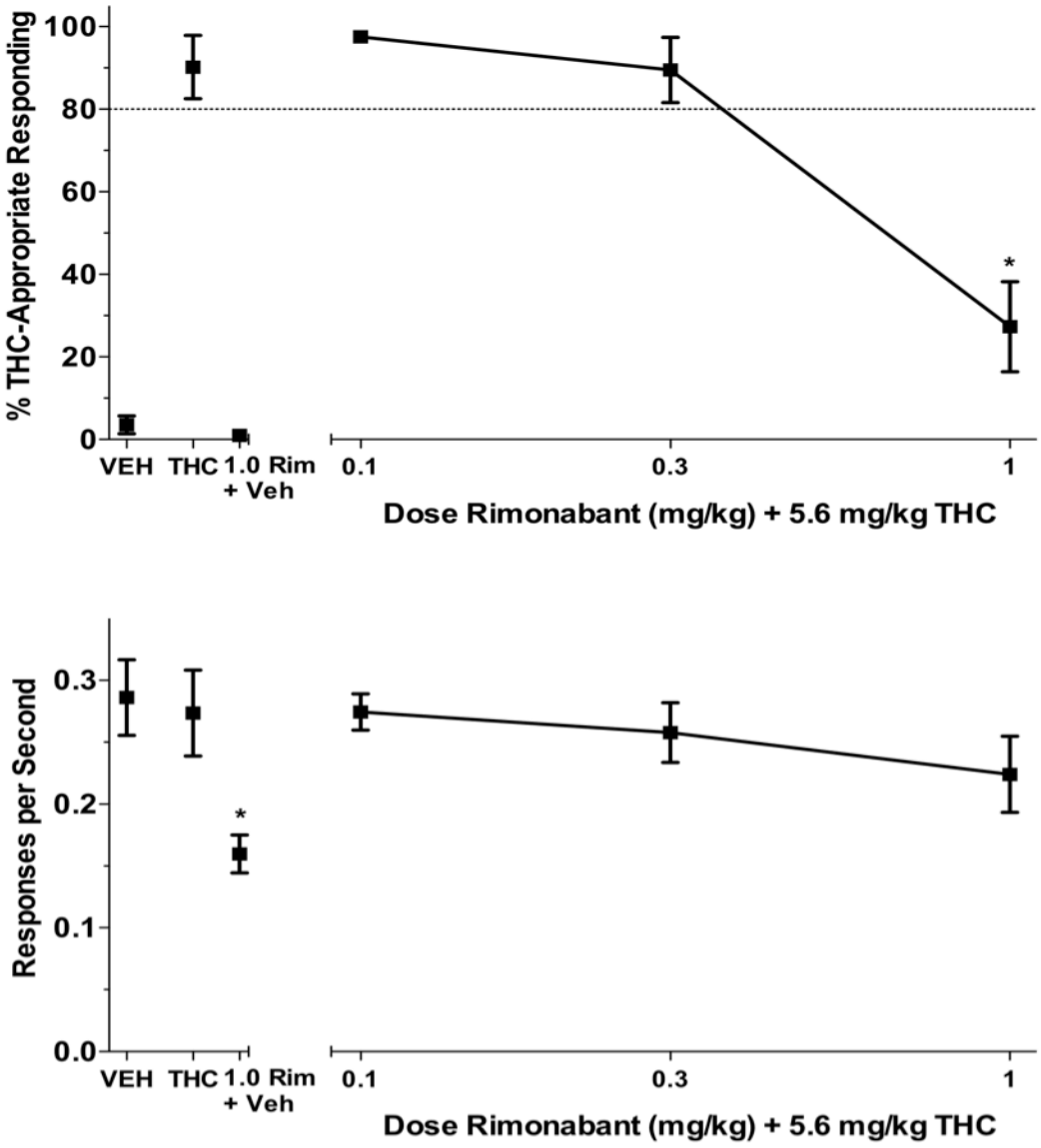


Figure 6. Rimonabant challenge. Effects of rimonabant in combination with the THC training dose on mean percentage THC-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 6). Values above 1.0 Rim + Veh represent control test data with 1.0 mg/kg rimonabant in combination with vehicle. For percentage THC-appropriate responding data, asterisks (*) denote significant differences compared to THC control, $p < 0.05$. All other details same as Figure 5.

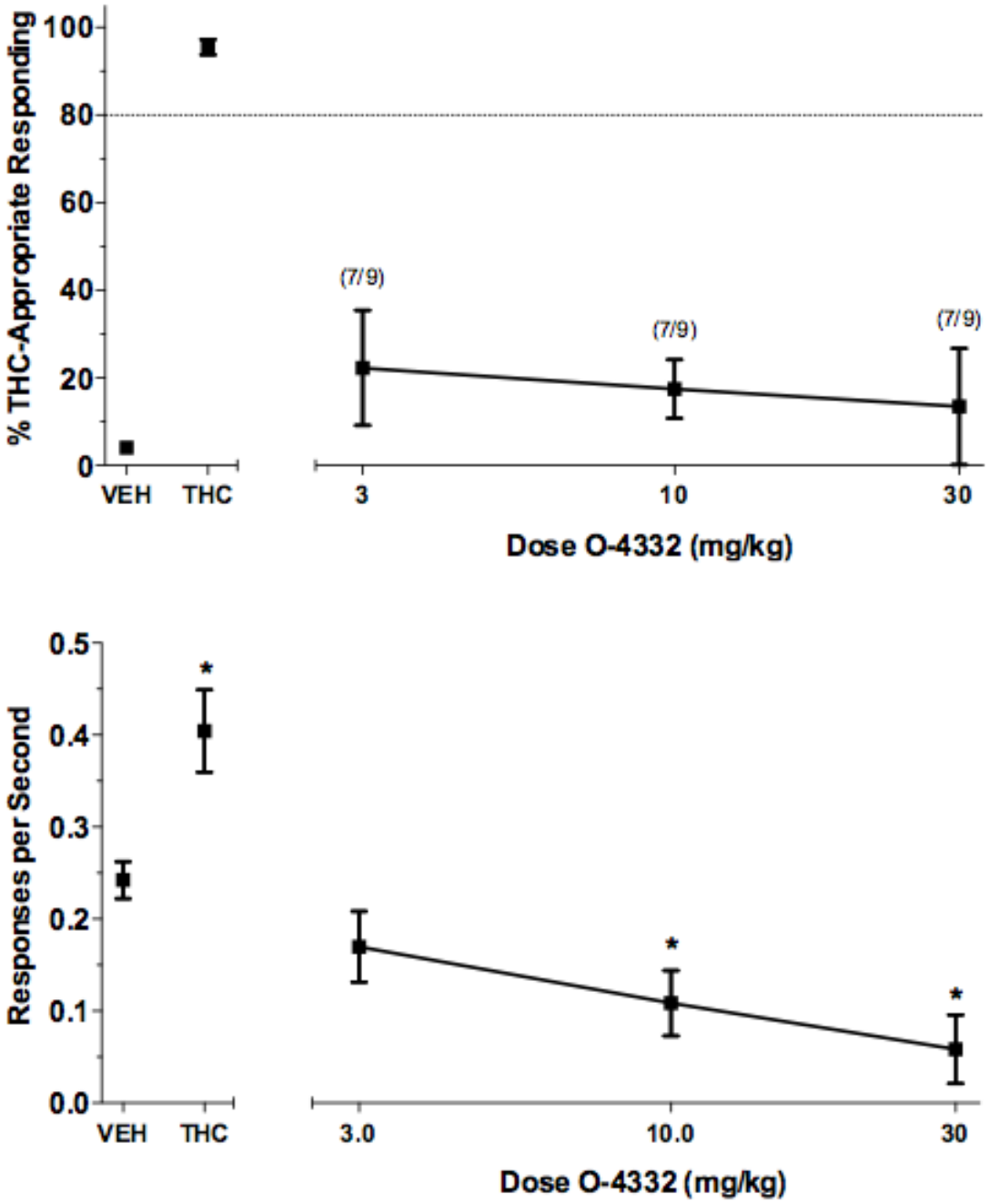


Figure 7. O-4332 generalization. Effects of O-4332 on mean percentage THC-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 9). All other details same as Figure 5.

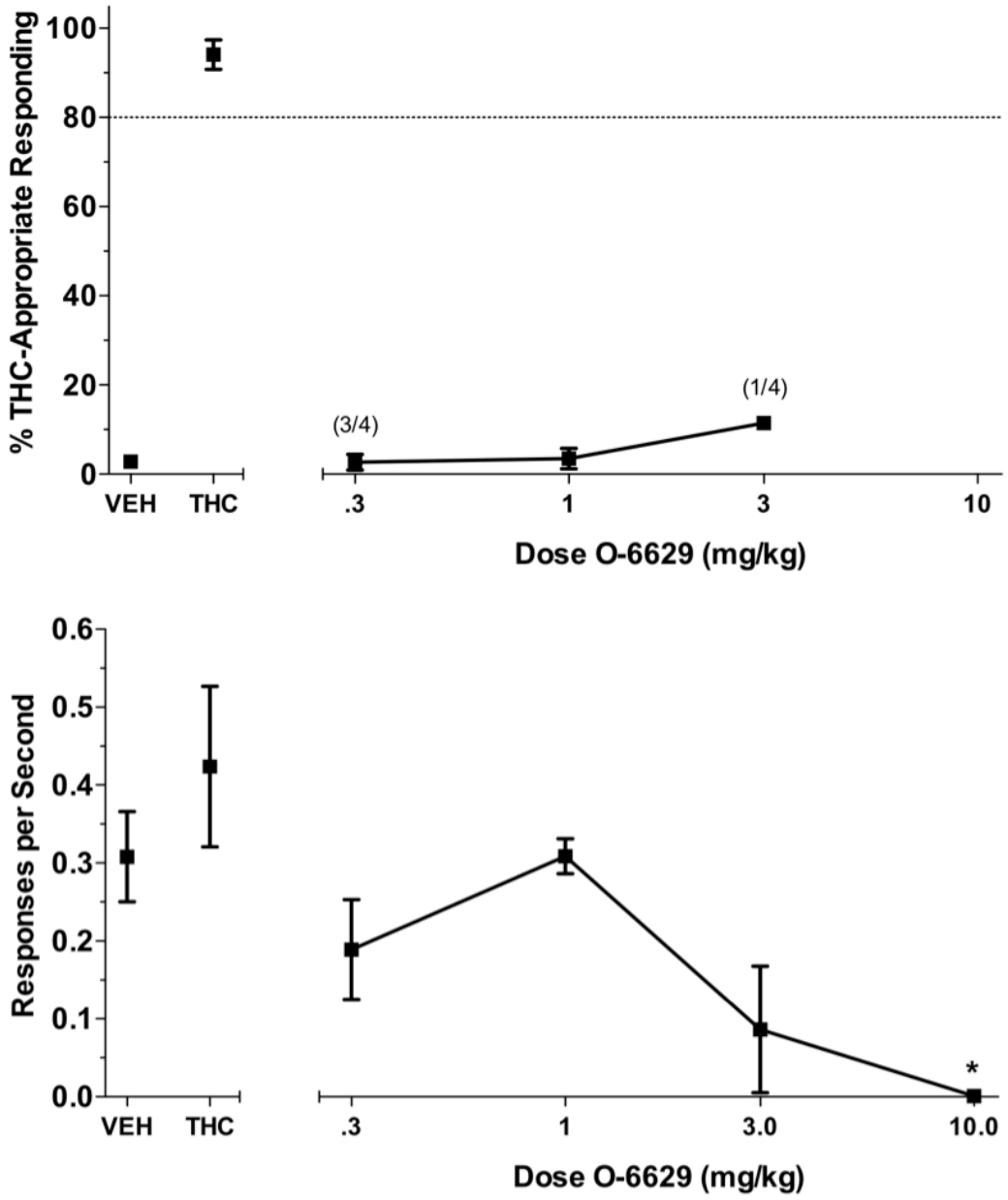


Figure 8. O-6629 generalization. Effects of O-6629 on mean percentage THC-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 4). All other details same as Figure 5.

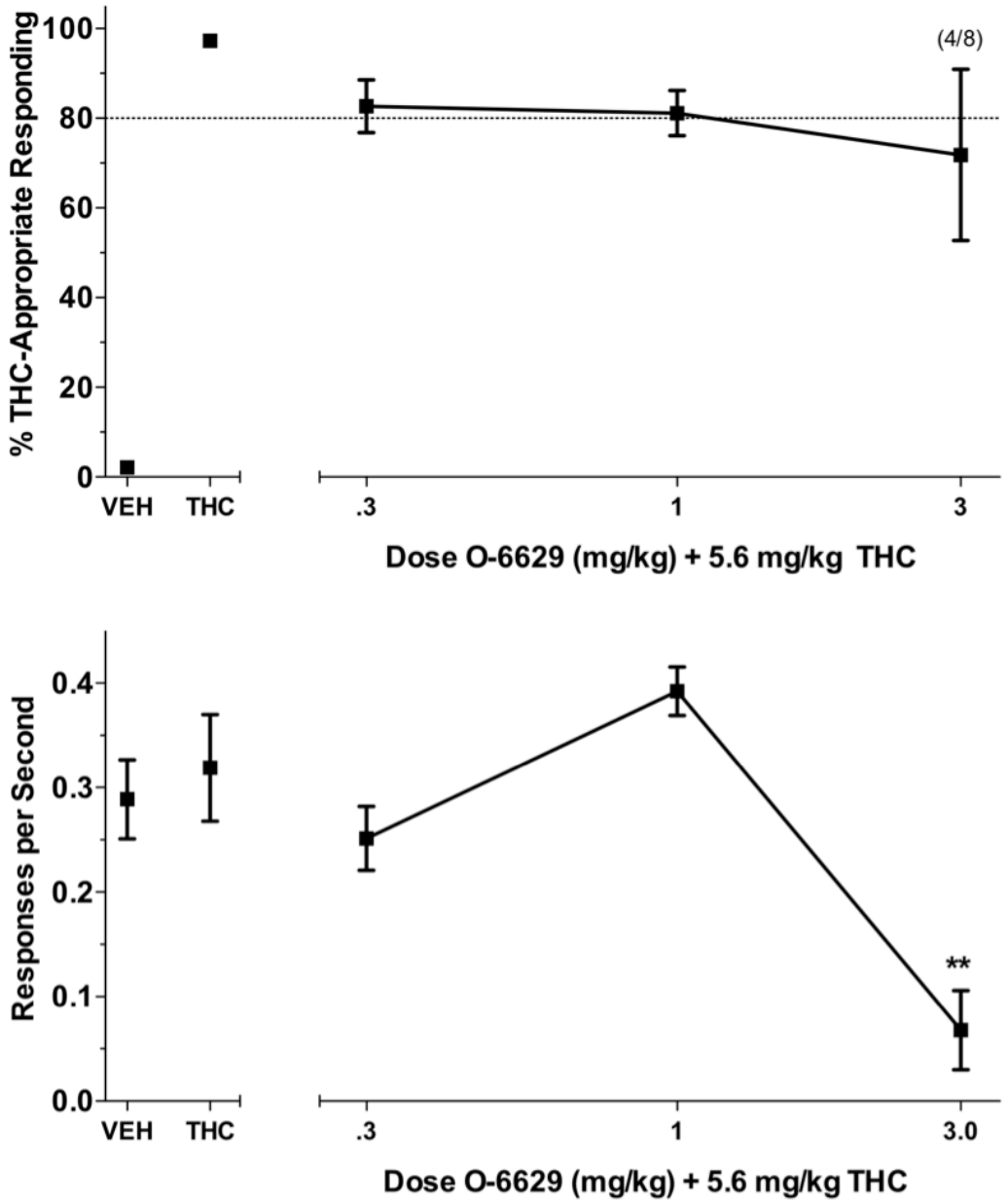


Figure 9. O-6629 challenge. Effects of O-6629 in combination with the THC training dose on mean percentage THC-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 8). All other details same as Figure 5.

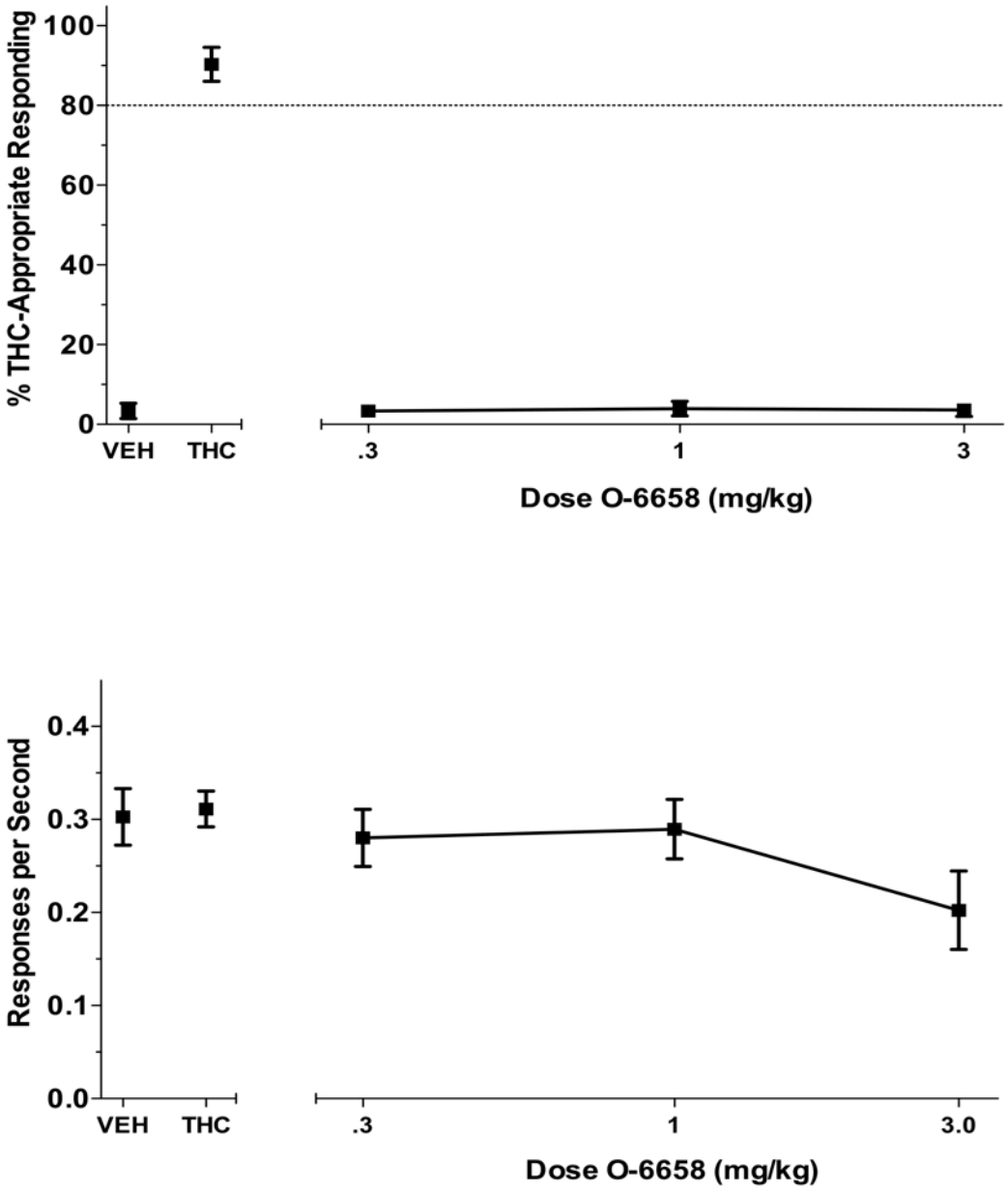


Figure 10. O-6658 generalization. Effects of O-6658 on mean percentage THC-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 6). All other details same as Figure 5.

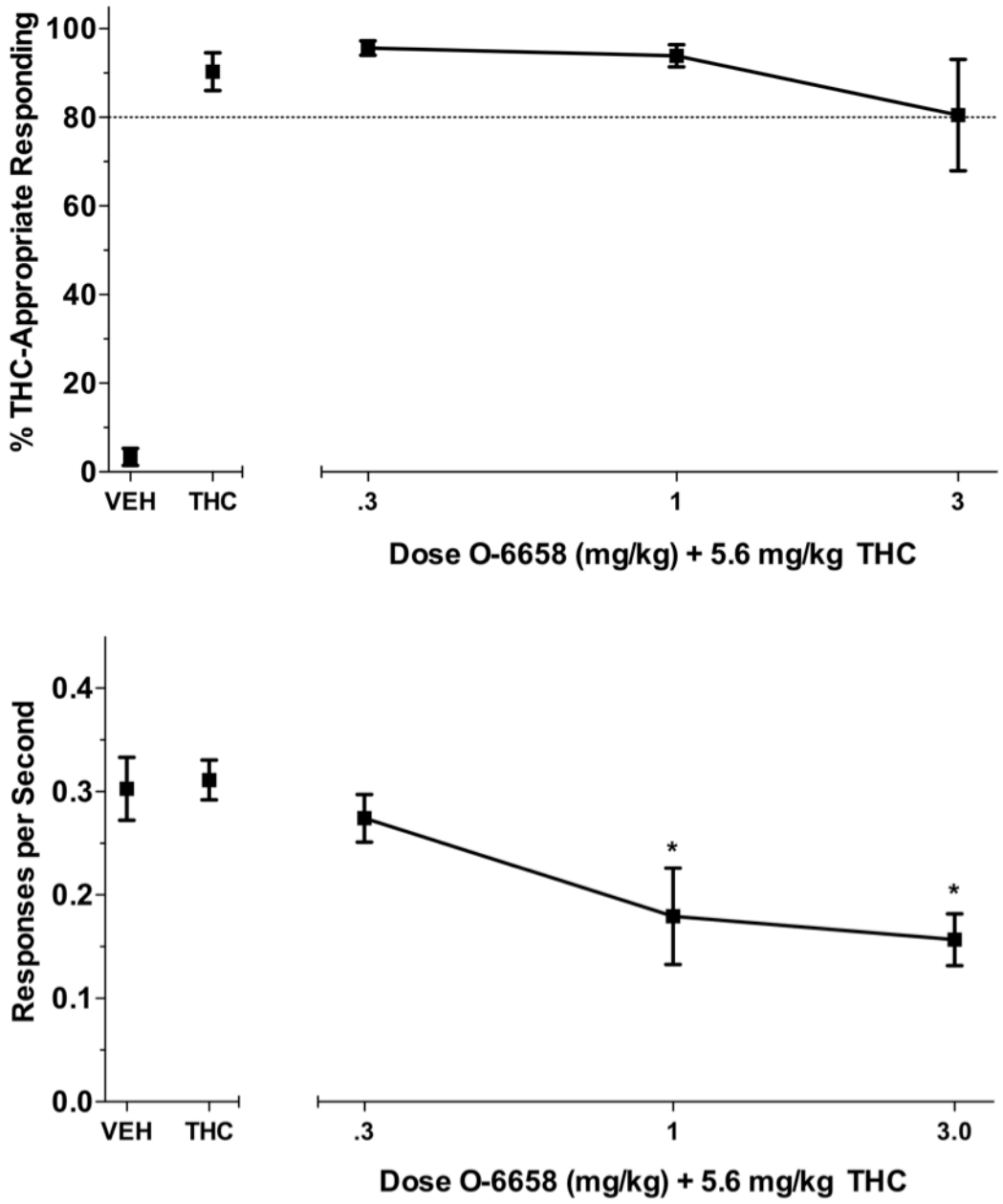


Figure 11. O-6658 challenge. Effects of O-6658 in combination with the THC training dose on mean percentage THC-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 6). All other details same as Figure 5.

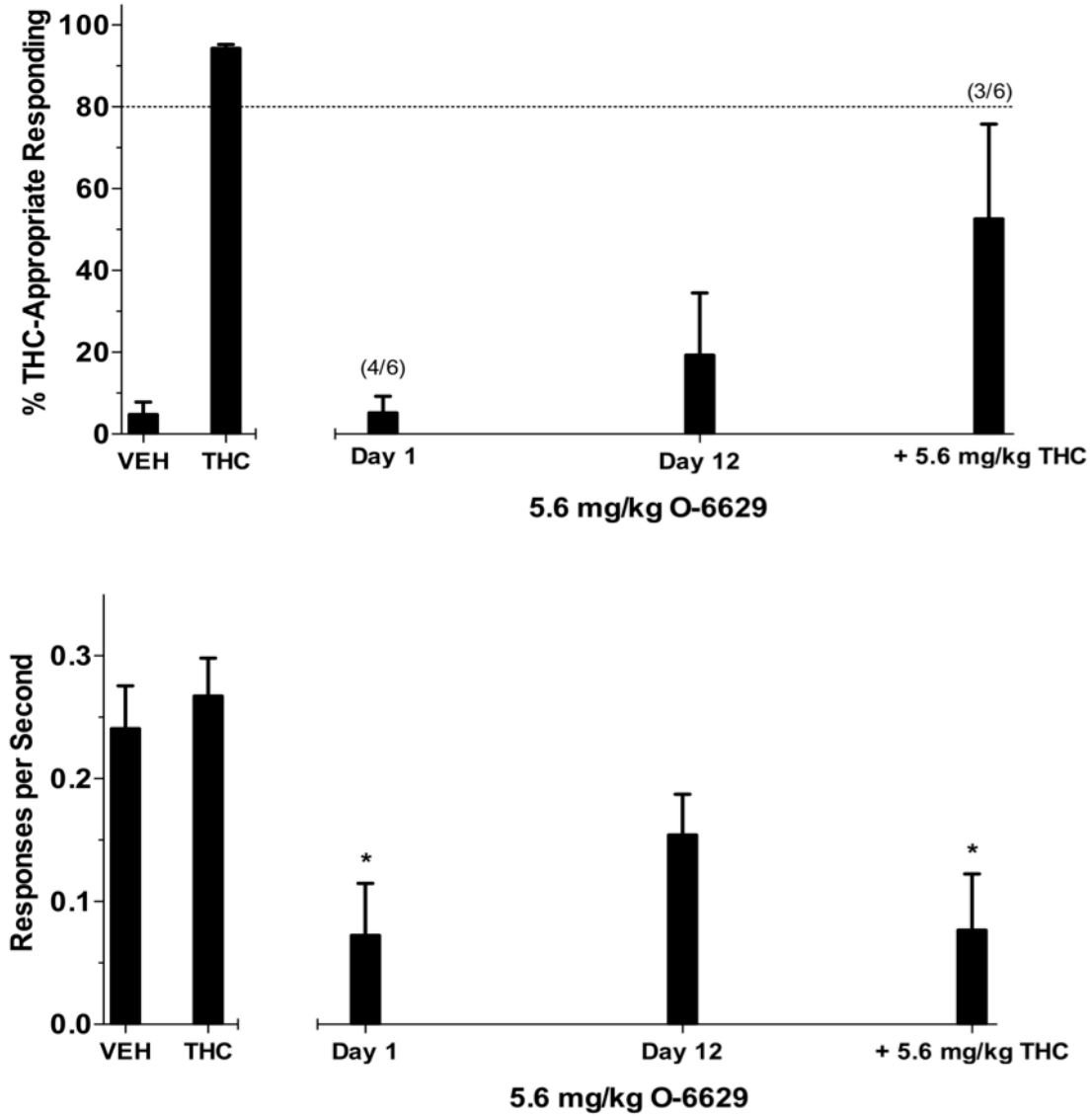


Figure 12. Evaluation of tolerance development to the rate suppressant effects of O-6629. Data shown represent mean percentage THC-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) following administration of 5.6 mg/kg O-6629 on days 1 and 12 and a challenge test against THC (N = 6). Points above VEH and THC refer to control tests conducted with vehicle and the training dose of THC on days 5 and 8. All other details same as Figure 5.

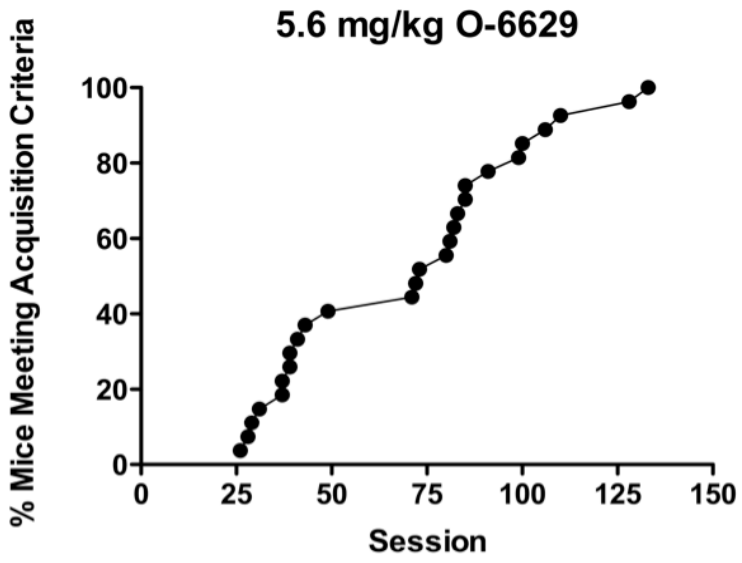


Figure 13. Acquisition of O-6629's discriminative stimulus. Values represent percentage of subjects meeting acquisition criteria across sessions (N = 27/31).

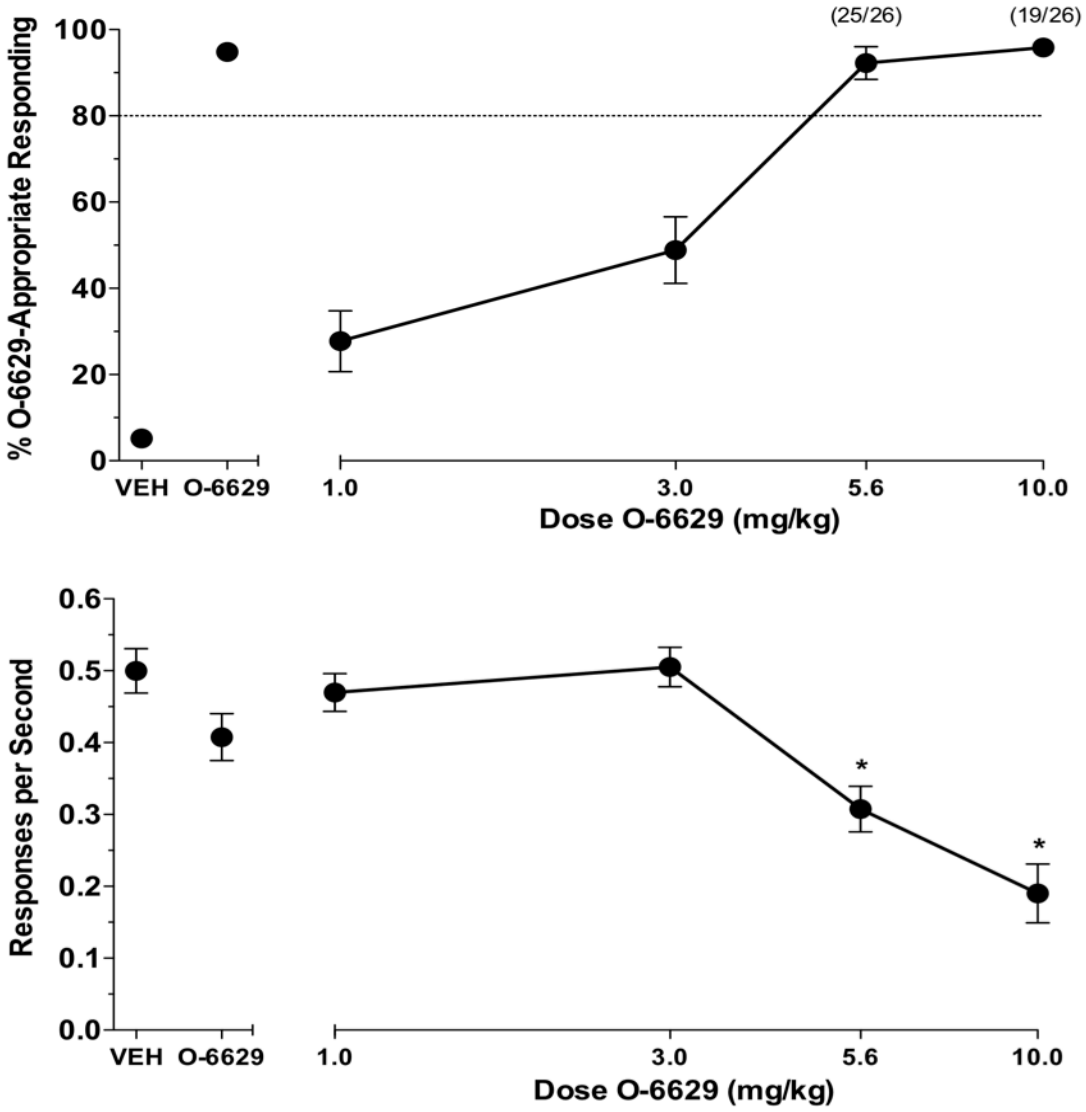


Figure 14. O-6629 generalization. Effects of O-6629 on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) in mice trained to discriminate 5.6 mg/kg O-6629 from vehicle (N = 26). Values above VEH and O-6629 represent control test data collected prior to conducting the dose effect determination. The dashed line at 80% drug lever responding indicates full generalization to the training dose. For response rate data, asterisks (*) denote significant differences compared to vehicle control, $p < 0.05$.

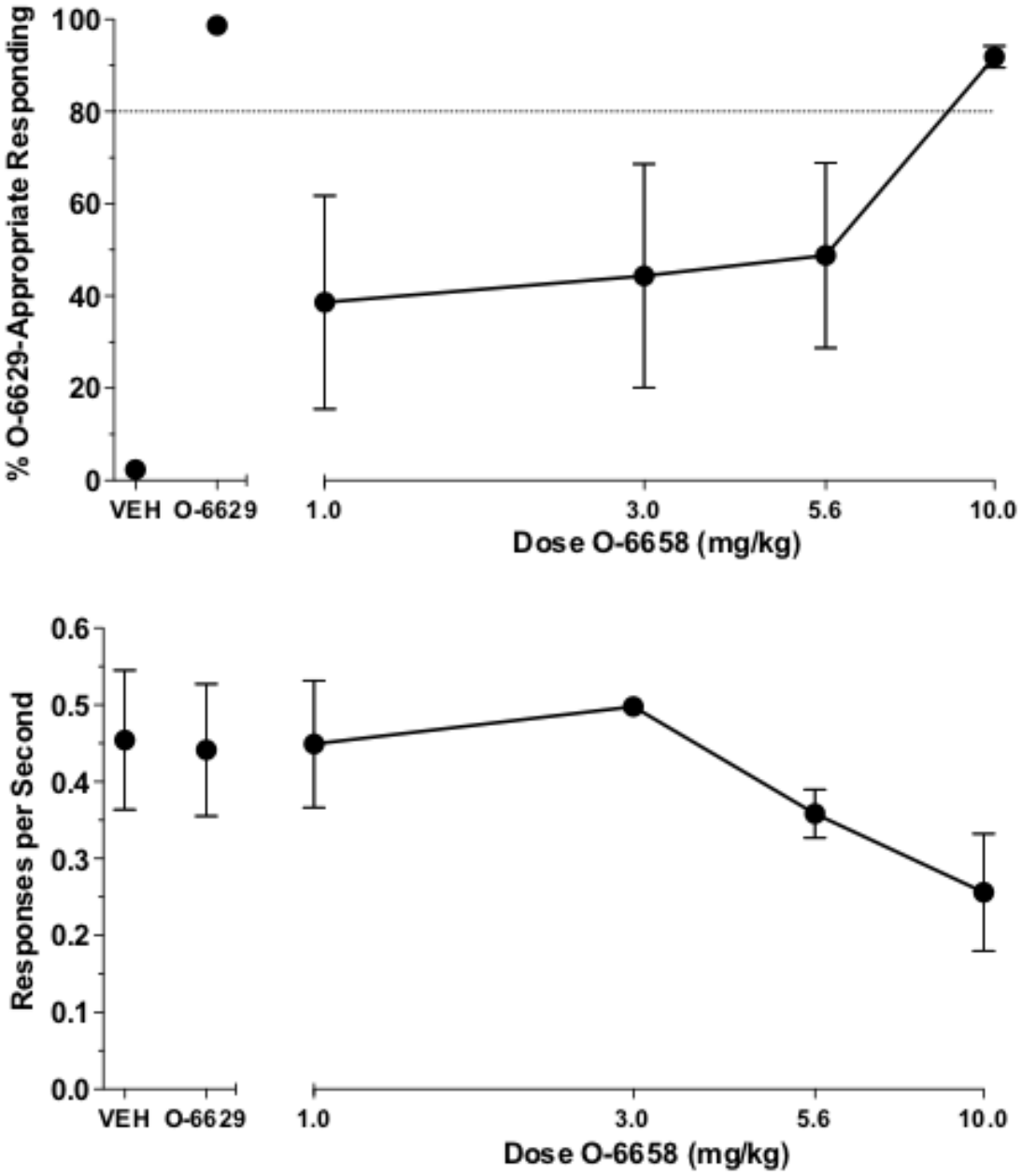


Figure 15. O-6658 generalization. Effects of O-6658 on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 4). All other details same as Figure 14.

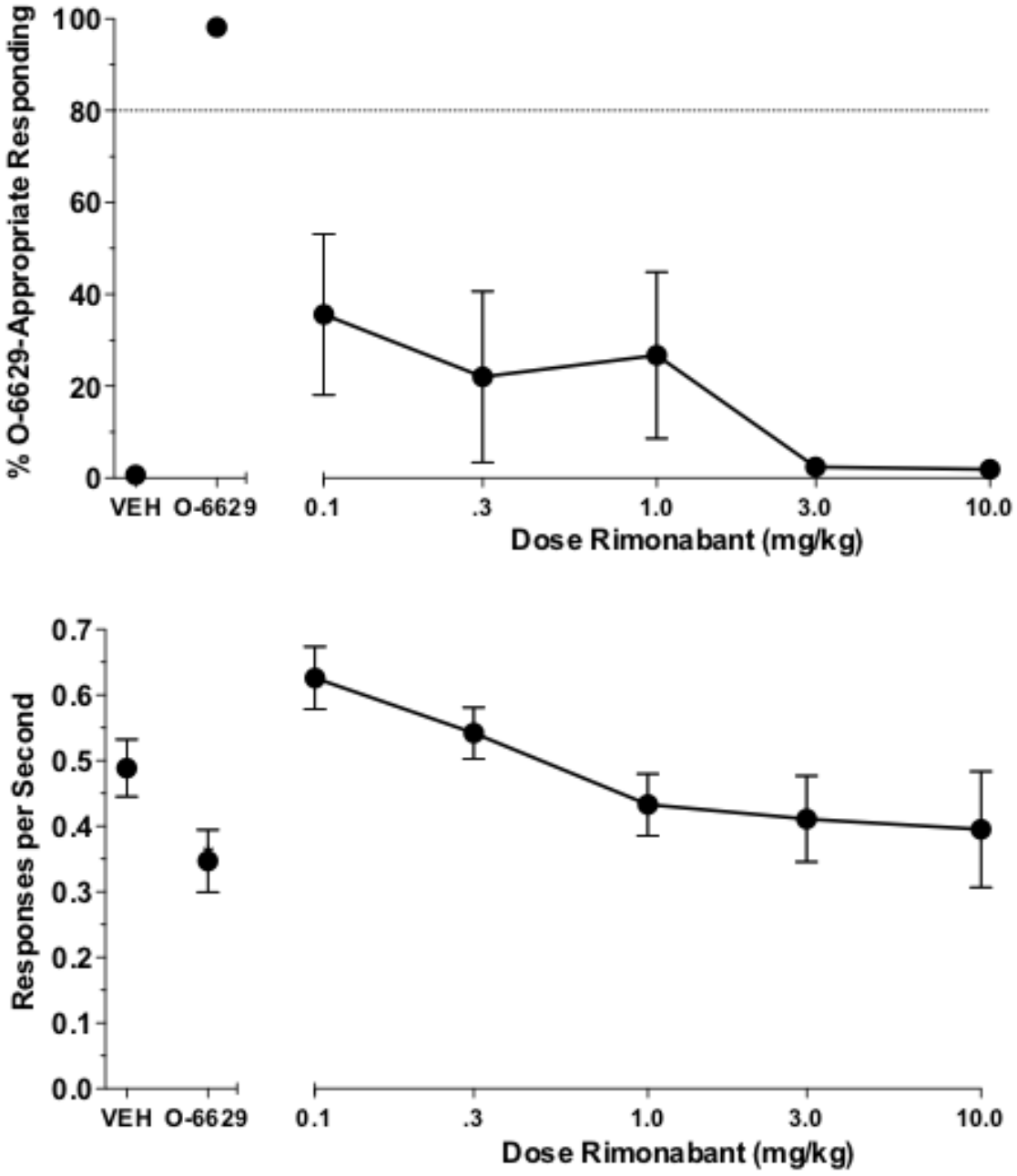


Figure 16. Rimonabant generalization. Effects of rimonabant on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 5). All other details same as Figure 14.

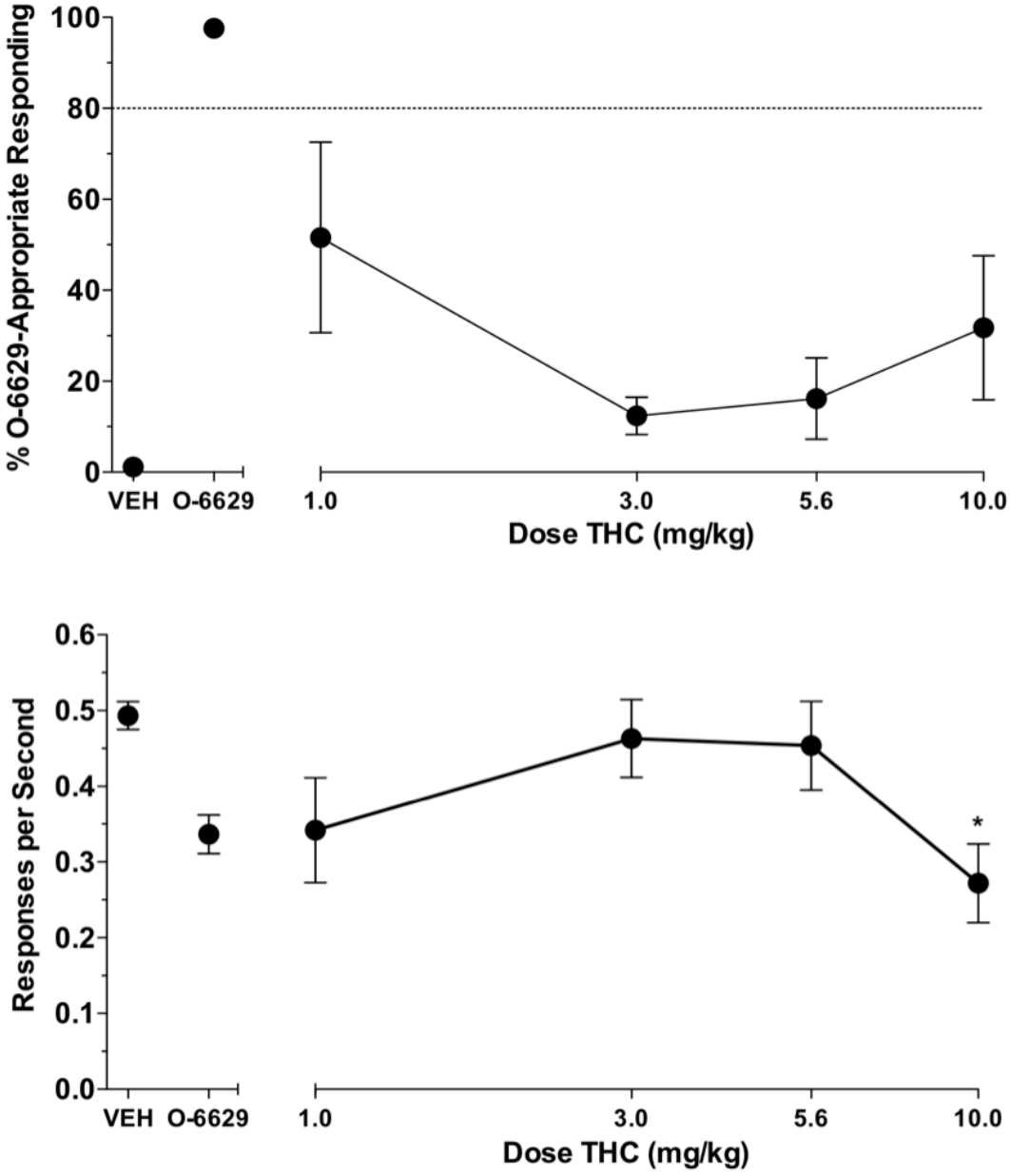


Figure 17. THC generalization. Effects of THC on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 6). All other details same as Figure 14.

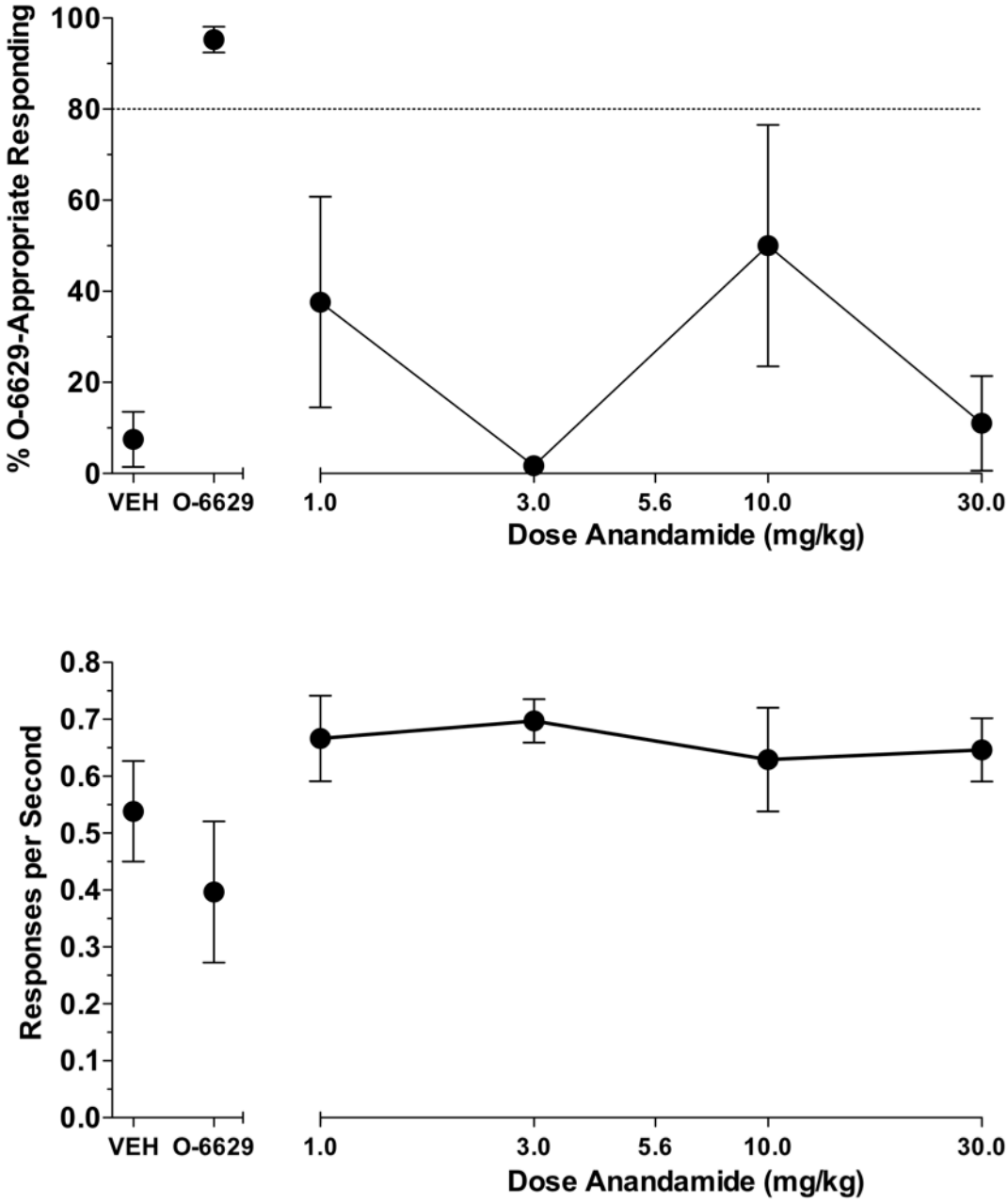


Figure 18. Anandamide generalization. Effects of anandamide on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 4). All other details same as Figure 14.

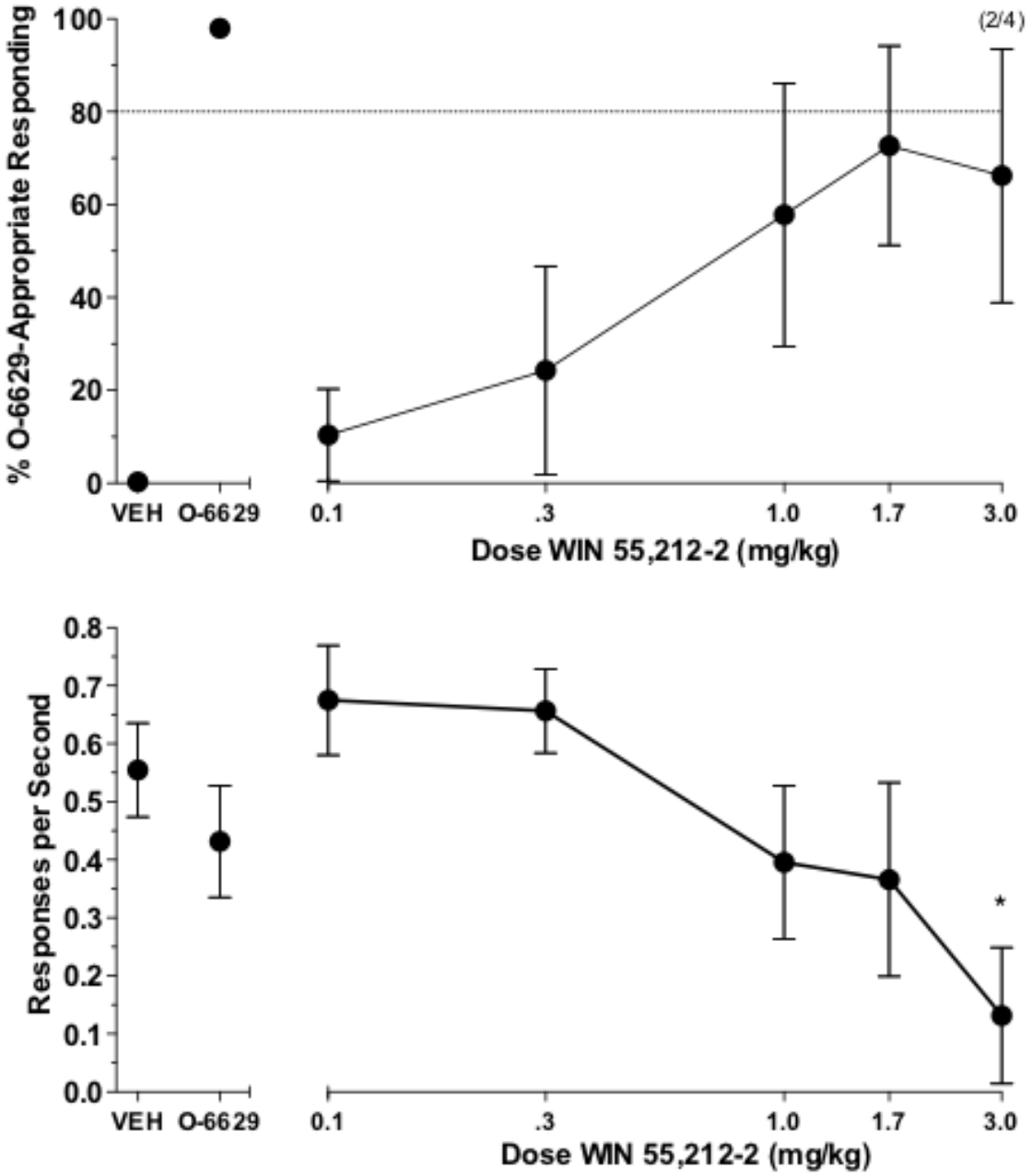


Figure 19. WIN 55,212-2 generalization. Effects of anandamide on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 4). All other details same as Figure 14.

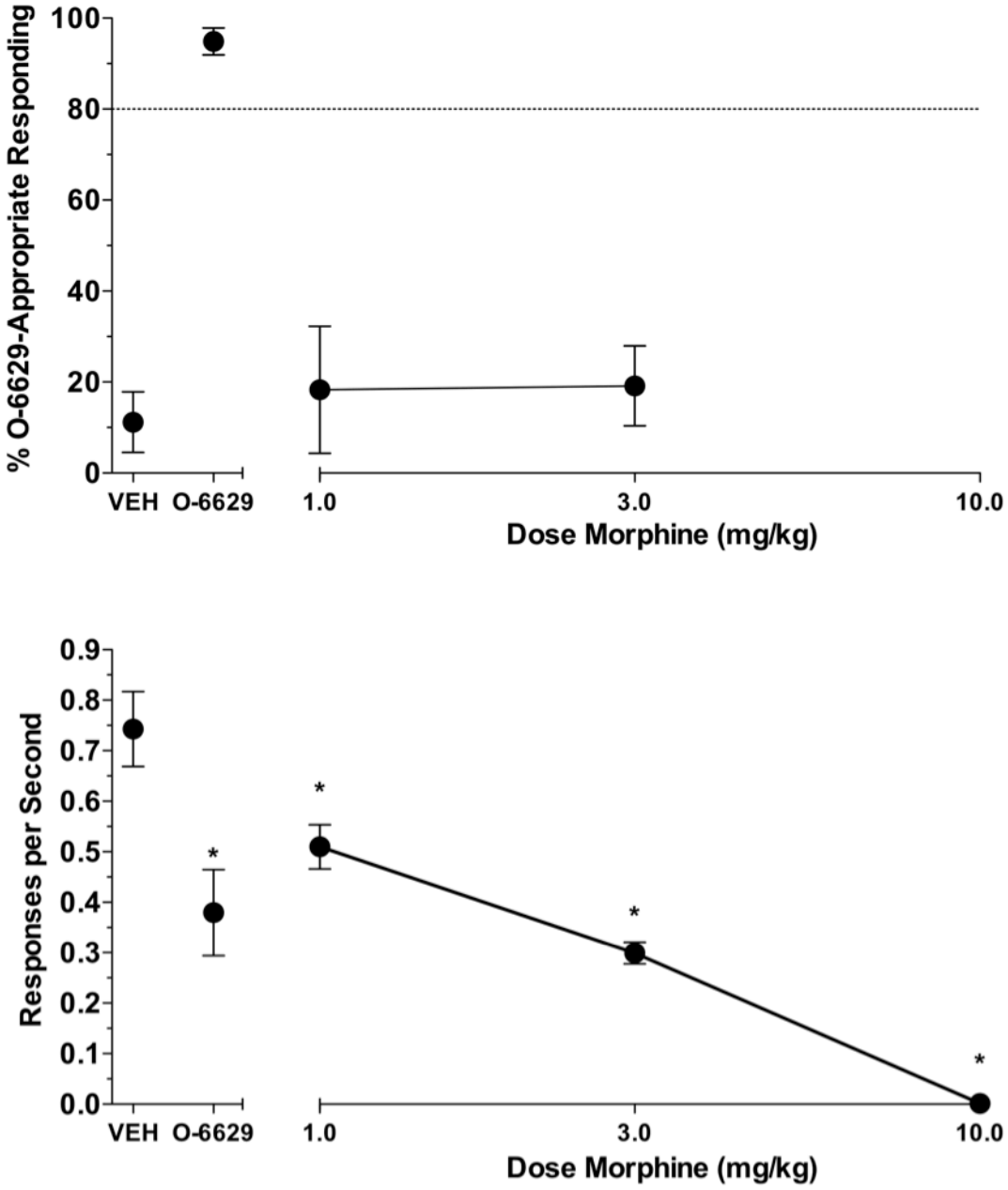


Figure 20. Morphine discrimination. Effects of morphine on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 7). All other details same as Figure 14.

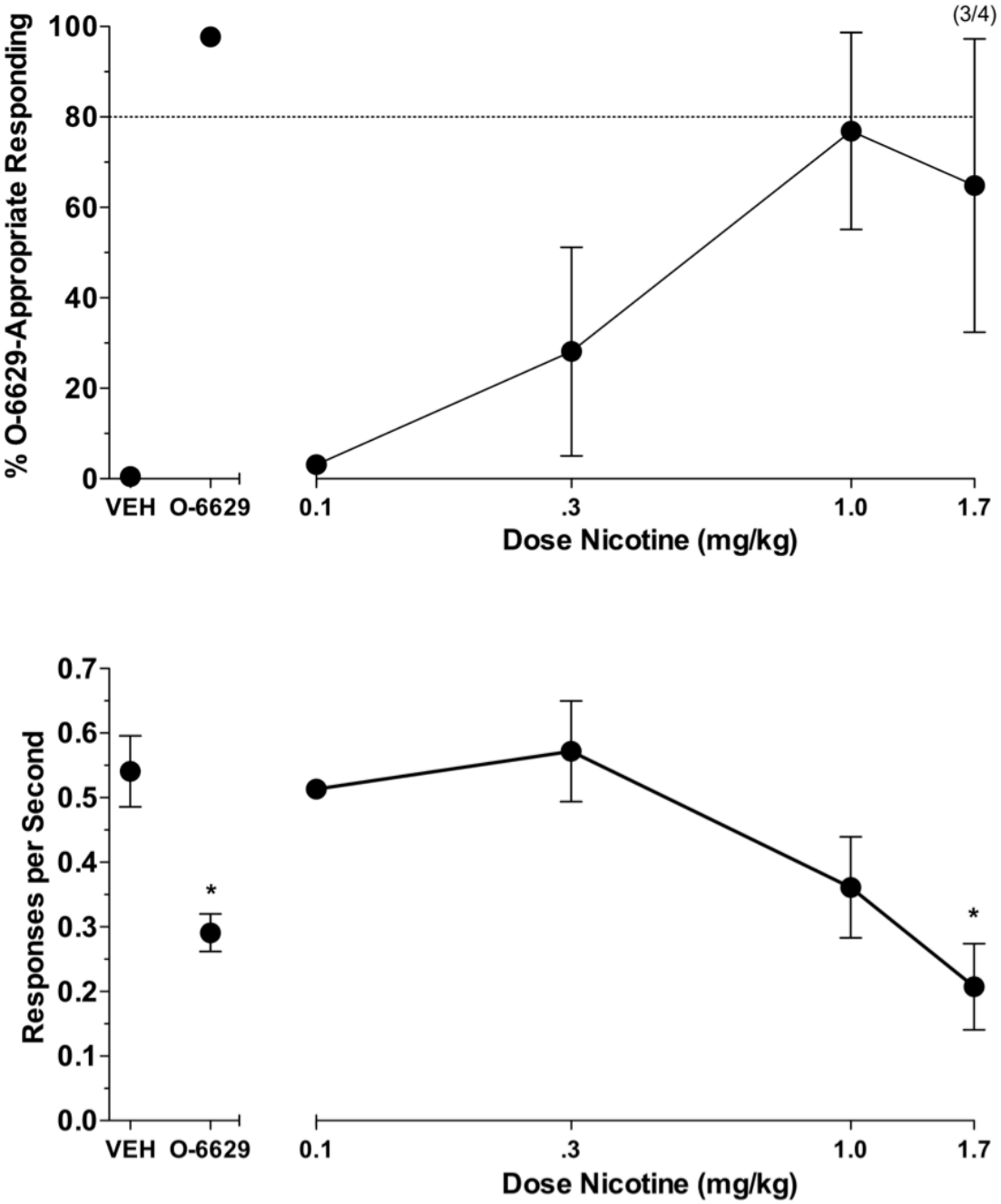


Figure 21. Nicotine generalization. Effects of nicotine on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 4). All other details same as Figure 14.

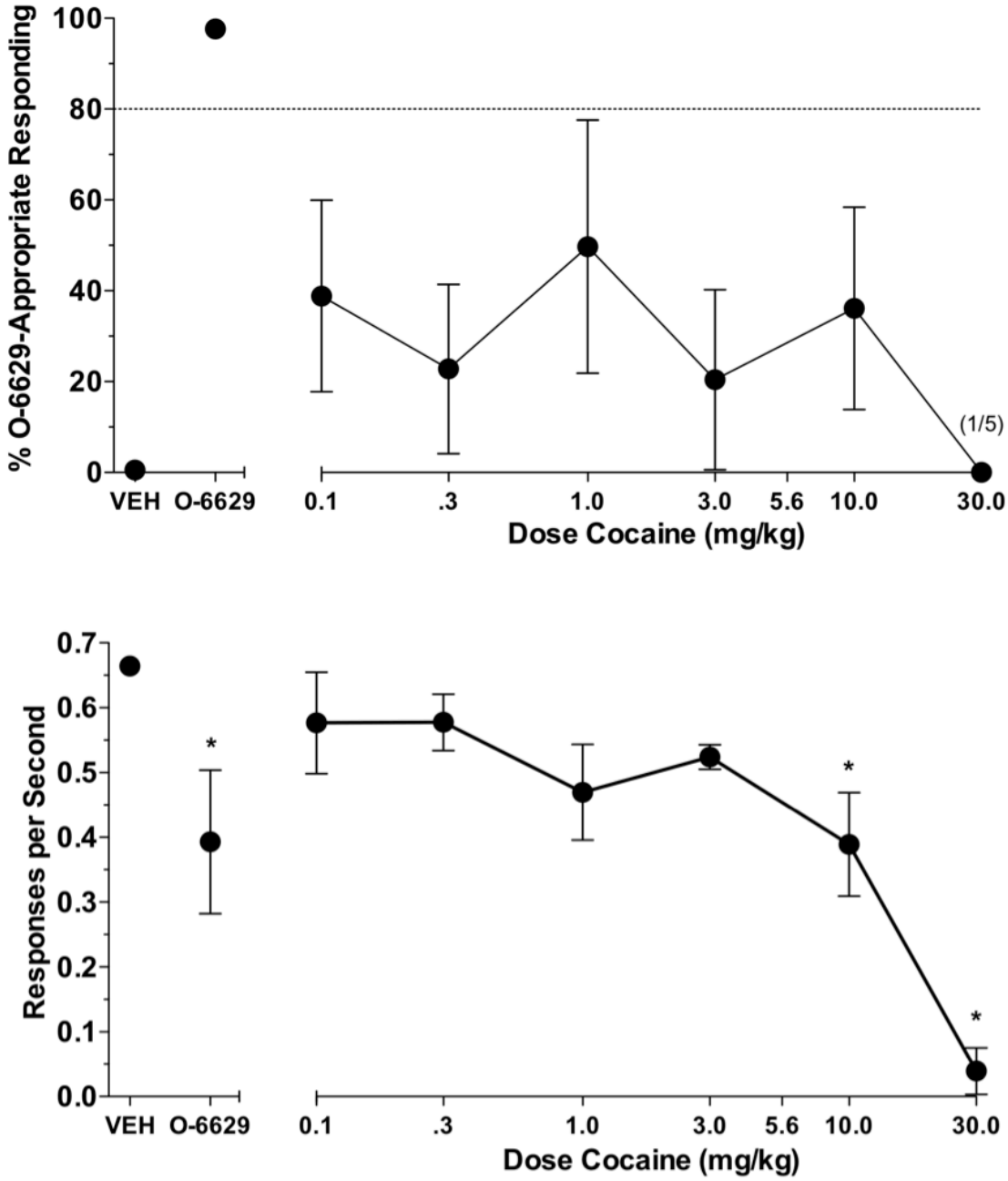


Figure 22. Cocaine generalization. Effects of cocaine on mean percentage O-6629-appropriate responding (±SEM; top panel) and mean responses per second (±SEM; bottom panel) (N = 5). All other details same as Figure 14.

Discussion

The present study evaluated the 3-substitutent rimonabant analogs O-6629 and O-6658 in two behavioral assays, the tetrad and drug discrimination. Activity in all four tests of the tetrad battery (Experiment 1) across a similar dose range is often predictive of cannabimimetic activity, whereas THC drug discrimination (Experiment 2) serves as a model for the psychoactive/intoxicating properties produced by marijuana in human users. In addition to comparing a discriminative stimulus of a drug with known mechanism of action to other drugs, the robust pharmacological specificity afforded by drug discrimination also permits training of drugs with unknown mechanism of action. In this case, O-6629 was trained as a discriminative stimulus (Experiment 3) and evaluated against drugs from several pharmacologically distinct classes with well-established mechanisms of action. The results of the present study indicate that O-6629 and O-6658 do not exert their effects through established cannabinoid mechanisms.

When evaluated in the tetrad battery, O-6629 produced significant decreases in spontaneous activity and body temperature, as well as significant antinociception and catalepsy. Like O-6629, O-6658 significantly decreased spontaneous activity and body temperature and produced catalepsy; however, a significant antinociceptive effect was not observed with O-6658. Although O-6629, and to a lesser extent O-6658, produced behavioral activity in the tetrad, these compounds differed from THC in regards to the maximal effects elicited by the latter. For example, THC reliably produces 4-6° C decreases in body temperature (Compton, Johnson, Melvin, & Martin, 1992; Wiley & Martin, 2003), which contrasts sharply with the 11° C decrease observed following O-6629 or O-6658 administration. In addition, catalepsy in THC treated mice typically ranges between 50-60% time immobile (Compton et al., 1992; Wiley & Martin, 2003). Maximal catalepsy produced by O-6629 and O-6658 was similar (28.28% and 32.11%, respectively). When tested with the high dose (30 mg/kg) of either compound, severe

loss of motor function was observed as demonstrated by tests of spontaneous activity test, and during the ring test, as all subjects were unable to remain positioned on the ring despite numerous trials of placing each mouse on the apparatus. A seizure was observed in one subject treated with 30 mg/kg O-6658. Administration of 3 mg/kg of either analog did not produce any behavioral activity, whereas significant decreases in spontaneous activity and body temperature were present with 10 mg/kg. Thus, 3 mg/kg doses of either analog would not be predicted to decrease operant response rates in drug discrimination as a result of motoric deficits.

Interestingly, the indole-derived potent CB1 agonist WIN 55,212-2 produces a behavioral profile rather similar to those elicited by the 3-substituent rimonabant analogs evaluated in this study, including 11° C decreases in body temperature, near complete suppression of spontaneous activity, and approximately 30% time immobile during assessment of catalepsy (Compton, Gold, Ward, Balster, & Martin, 1992). Despite these similarities, the 3-substituent rimonabant analogs evaluated here differed from WIN 55,212-2 and other cannabinoid agents in terms of their ability to produce antinociception. Whereas THC, WIN 55,212-2 and a variety of other cannabinoid agonists produce a full antinociceptive effect (e.g., 10 s latency in tail flick; 100% MPE), O-6629 and O-6658 produced 42% and 23% MPE, respectively. One possible explanation for the diminished antinociceptive effect seen with the rimonabant analogs is pharmacokinetic factors, as intravenous administration of these compounds produced a maximal antinociceptive effect (JL Wiley, unpublished data). Additionally, the overwhelming majority of tetrad determinations have utilized intravenous or intraperitoneal route of administration, whereas the subcutaneous injections were performed for these studies. Another key consideration is that C57BL/6 mice were used in the present study, whereas ICR mice have typically served as subjects in the tetrad in this laboratory.

Several other drug classes have been shown to produce cannabimimetic activity in the tetrad through a non-CB1 mechanism. As mentioned previously, some antipsychotic drugs, such as chlorpromazine displayed a cannabimimetic profile in the tetrad that was not reversed by rimonabant (Wiley & Martin, 2003). The endogenous cannabinoid anandamide also produced activity in the tetrad, but was not reversed by rimonabant (Adams, Compton, & Martin, 1998). Further, tetrad effects were present following anandamide administration in CB1 knockout mice (Di Marzo et al., 2000b). A series of investigations on arvanil, a hybrid cannabinoid and vanilloid derived from the chemical structures of anandamide and capsaicin (Melck et al., 1999) provided further support for the possible existence of novel receptors that mediate cannabinoid activity. When evaluated in the tetrad, arvanil produced tetrad activity similar to anandamide (i.e., THC-like responses for spontaneous activity, antinociception and catalepsy; 3°C decreases in body temperature) that was not reversed by rimonabant (Di Marzo et al., 2000a). Arvanil was also more potent than capsaicin in generating antinociception, but arvanil's antinociceptive properties were not blocked by the TRPV1 antagonist capsazepine, whereas capsaicin-induced antinociception was reversed (Di Marzo et al., 2000a).

In sum, the tetrad has served as a tremendously valuable screen for assessing cannabinoid activity in vivo. A drug is likely to possess cannabinoid activity when it: 1) produces activity in all four tests to a comparable magnitude as cannabinoid agonists (i.e., approximately 90% suppression of spontaneous activity, 100% MPE, Δ -6° C, 60% time immobile) and 2) does so within a similar dose range between all four tests. The 3-substitutent rimonabant analogs evaluated in this study were active in the tetrad, but to a different degree than the prototypic cannabinoid THC. Combined with earlier findings demonstrating rimonabant's inability to reverse these effects, as well as their presence in CB1 knockout mice, it can be concluded that

the activity produced by these 3-substituent rimonabant analogs is mediated through a non-CB1 mechanism. Interestingly, these analogs actually produced a behavioral profile more similar to WIN 55,212-2 than THC, suggesting that these analogs might be acting through a novel cannabinoid mechanism. This possibility will be discussed later.

THC (5.6 mg/kg) readily served as a discriminative stimulus in mice, with subjects meeting acquisition criteria in a mean of 29.50 sessions. Dose-dependent generalization to THC's discriminative stimulus was observed as is typical for studies of this nature, with partial substitution occurring at the 3 mg/kg dose. None of the 3-substituent rimonabant analogs evaluated produced full or partial substitution for THC, providing evidence that these analogs do not produce THC-like psychoactivity.

Although THC discrimination has been established in mice only recently, the pharmacological specificity of this model in the species has been demonstrated by findings from our laboratory and others. The structurally dissimilar synthetic agonists CP55,940 and WIN 55,212-2 both substitute for THC (McMahon et al., 2008), as does the endocannabinoid anandamide in the presence of a FAAH inhibitor (Vann et al., 2009a). On the contrary, non-cannabinoids (e.g., cocaine, ethanol and ketamine) do not elicit THC-like responding (McMahon et al., 2008). THC discrimination in mice has also been used in combination with other in vivo and in vitro techniques as a mechanistic tool for evaluating a putative neutral CB1 receptor antagonist (Wiley et al., 2011) and salvinorin A, the psychoactive constituent of *Salvia divinorum* (Walentiny et al., 2010).

In the present study, THC's discriminative stimulus was dose-dependently attenuated by rimonabant, in accordance with a number of reports demonstrating CB1 receptor mediation of THC's discriminative stimulus in mice (McMahon et al., 2008; Vann et al., 2009a) and other

species (Wiley et al., 1995b; Mansbach et al., 1996). Results from challenge tests with O-6629 and O-6658 revealed that these compounds failed to markedly attenuate THC's discriminative stimulus. Although a challenge with 3 mg/kg O-6629 decreased THC-appropriate responding below full substitution criteria (i.e., < 80% THC-appropriate responding), it should be noted that full substitution was observed in 3 out of 4 mice responding during that test session. Response rates were severely suppressed (i.e., < 10 total responses) for the remaining 4 mice and their generalization data were subsequently excluded from analysis. Thus, despite their chemical derivation and CB1 receptor affinity, it is unlikely that O-6629 and O-6658 are acting as functional antagonists of CB1 receptors.

Although these compounds failed to modify the training dose of THC, it is possible that these 3-substituent rimonabant analogs might have produced shifts in the THC dose effect curve. A number of agonists from distinct pharmacological classes (e.g., μ-opioid, acetylcholine, dopamine) have been shown to shift the THC dose effect curve leftward through a non-cannabinergic mechanism in rats (Solinas & Goldberg, 2005a; Solinas et al., 2007a; Solinas et al., 2010). Interestingly, these drugs did not produce substitution when given alone, nor did their respective antagonists attenuate the discriminative stimulus of the THC training dose. These findings, and others (e.g., partial substitution for THC produced by diazepam Wiley & Martin, 1999) clearly demonstrate that a number of neurochemical systems can influence the discriminative stimulus effects of THC and may be attributable to co-localization of CB1 receptors with other neurotransmitter systems.

Due to limited drug supply, O-4332 was only evaluated for generalization to THC's discriminative stimulus. Similar to the other 3-substituent analogs, O-4332 did not substitute for THC. Although a challenge test was not conducted with O-4332, it is unlikely that O-4332

would have modified THC's discriminative stimulus. The weak CB1 receptor affinity (relative to the other analogs tested; Table 1), and lack of observed effect with other 3-substituent rimonabant analogs on THC's discriminative stimulus support this hypothesis.

Given the lack of apparent behavioral activity of 3 mg/kg O-6629 in the tetrad compared to the robust behavioral effects seen with 10 mg/kg, 5.6 mg/kg O-6629 was chosen as the initial target dose to train as a discriminative stimulus. Results obtained in THC-trained mice demonstrated development of tolerance to the rate suppressant effects of 5.6 mg/kg O-6629 following 12 days of once daily chronic treatment, lending further evidence that this dose would be suitable for training as a discriminative stimulus.

Attempts to train 5.6 mg/kg O-6629 as a discriminative stimulus were successful, with subjects acquiring the discrimination in a mean of 69.56 sessions. Acquisition of O-6629's discriminative stimulus took appreciably longer than THC. Several possible factors contributed to this, including a longer time to develop tolerance to the rate suppressant effects of O-6629 relative to THC. Indeed, response rates during O-6629 control tests were often, but not always, lower than vehicle control rates. Additionally, experimenter history could account for some of the discrepancy in latency to meet acquisition criteria, as our laboratory has had extensive experience training THC as a discriminative stimulus, but O-6629 has never been trained in this procedure. Nonetheless, the ability of O-6629 to serve as a traditional discriminative stimulus (i.e., drug vs. non-drug discrimination without other manipulations) differentiates it from rimonabant. The fact that systemically administered O-6629 trained as a discriminative stimulus also provides strong behavioral evidence that O-6629 penetrates the blood brain barrier, as peripherally restricted drugs fail to serve as discriminative stimuli.

Although the present study did not evaluate whether other doses of O-6629 would serve as a discriminative stimulus, it is likely, albeit speculative, that the rate suppressant effects of O-6629 would limit training efforts of a higher dose as a discriminative stimulus. Rate suppression is frequently observed during initial dosing in drug discrimination procedures and typically diminishes over the course of training as a result of frequent (but not chronic) exposure to the same drug dose. Other methodological manipulations (e.g., initial chronic exposure as opposed to double alternation) might have facilitated acquisition of O-6629's discriminative stimulus.

Also unclear is whether lower doses of O-6629 would serve as a discriminative stimulus. While 3 mg/kg O-6629 did not produce any activity in the tetrad, this dose did disrupt operant responding in mice discriminating THC. Training dose has been shown to be a critical determinant of a drug's ability to function as a discriminative stimulus and different training doses can result in different substitution patterns with test compounds (Picker et al., 1993; Jarbe et al., 1998; Porter, Varvel, Vann, Philibin, & Wise, 2000; Jarbe et al., 2000). Thus, the pharmacological specificity of a drug's discriminative stimulus has the potential to be modified as a function of training dose.

O-6658 fully substituted for O-6629, providing further evidence that these two analogs share a common mechanism of action. Also, no significant rate suppression was observed with the dose of O-6658 (10 mg/kg) that substituted for O-6629. The parent compound rimonabant (0.1-10 mg/kg) did not engender meaningful O-6629-like responding, nor did it perturb operant response rates. This latter finding is particularly interesting, given rimonabant's well-established ability to diminish food intake. In the current study, 1 mg/kg rimonabant significantly decreased response rates when given alone, but not in combination with THC (experiment 2). This discrepancy might be indicative of some cross-tolerance between the appetite disrupting effects

of rimonabant and O-6629, but other interpretations are possible. For instance, vehicle response rates were lower in the THC-trained mice during rimonabant tests compared to O-6629 mice (0.29 vs. 0.49 responses/s). Also unclear is to the influence O-6629 or similar analogs have on feeding behavior, as will be discussed in further detail later.

Significant rate suppression was observed during O-6629 control tests for the morphine and cocaine dose effect determinations, suggesting only a partial degree of tolerance development to the rate suppressant effects of 5.6 mg/kg O-6629 over the course of the experiment. In the present study, 3 mg/kg O-6629 severely suppressed response rates (< 10 total responses during test session) in 3 out of 4 subjects in the THC discrimination experiment. However, the mean response rate at this dose was not significantly different from vehicle, likely attributable to small sample size. It is currently unknown whether O-6629 or similar analogs would decrease food intake at doses that do not suppress locomotor activity, but these preliminary findings and rimonabant's known effects on food intake suggest this could be a possibility.

Another possibility is that these analogs might decrease motivation to respond for a food reinforcer. One approach to investigate this would be to evaluate the effects of these analogs in animals responding under a food-reinforced progressive ratio schedule, whereby behavioral requirements for food reinforcement increase systematically (e.g., FR1, FR2, FR4, FR8, etc.) over the course of an experimental session (Hodos, 1961). Drug treatment that results in a subject extinguishing responding at a final fixed ratio schedule (referred to as breakpoint) lower than vehicle would be indicative of a decrease in the subject's motivation to respond for food reinforcement. Preclinical investigations of rimonabant's effects on food-reinforced PR breakpoints have yielded mixed findings (Solinas & Goldberg, 2005b; Maccioni, Pes, Carai,

Gessa, & Colombo, 2008; Ward & Walker, 2009), which may be explained in part by methodological variations between studies. Co-evaluations of O-6629 and other analogs in measures of non-contingent feeding behavior, fixed ratio operant responding, progressive ratio operant responding and locomotor activity would serve as an initial attempt to dissociate between locomotor versus potential appetitive and/or motivational alterations these compounds may produce.

THC failed to produce O-6629-like discriminative stimulus effects, even at doses that suppressed operant response rates. The greatest degree of O-6629-like responding occurred at the lowest dose tested (1 mg/kg). Full substitution was observed in half the subjects receiving this dose, but this effect did not persist during tests with higher doses. In the event that O-6629's discriminative stimulus was mediated totally or in part by cannabinergic activity, one would expect increases in O-6629-appropriate responding at higher THC doses. Taken together with results obtained in mice discriminating THC, there is no evidence to suggest that O-6629 produces cannabis-like intoxication.

Similarly, anandamide did not substitute for O-6629 but failed to decrease rates at doses tested (1-30 mg/kg). These results are not surprising, provided that anandamide was given in the absence of a metabolic inhibitor (e.g., URB597, PF-3845). Like THC, a moderate amount of O-6629-appropriate responding (~50%) was observed at one dose tested (10 mg/kg), whereby substitution was observed in half of the subjects tested. Any potential trend towards substitution was not observed during tests with lower or higher doses.

In contrast to these results obtained with centrally active cannabinoids, several doses of WIN 55,212-2 (1.7 and 3.0 mg/kg) produced partial substitution for O-6629's discriminative stimulus. Although the precise mechanism of action for O-6629 and other related analogs has not

yet been determined, these compounds may be acting through an, as of yet, undetermined cannabinoid mechanism. Indeed, mounting behavioral and neurochemical evidence from tests with anandamide and WIN 55,212-2 suggest the possible existence of [a] novel cannabinoid receptor/s. Anandamide was first shown to produce activity in the tetrad similar to THC, yet these effects were not reversed by rimonabant (Adams et al., 1998). An evaluation of cannabinoid effects on food-maintained operant responding in CB1 wild-type and knockout mice revealed that THC dose-dependently decreased operant responding in wild-type mice, but had no influence on response rate in knockout mice (Baskfield, Martin, & Wiley, 2004). However, methanandamide, an analog of anandamide with increased metabolic stability, decreased response rates to a similar degree in both genotypes (Baskfield et al., 2004). Somewhat surprisingly, this effect did not carry over during tests with O-1812, a methanandamide analog that produces THC-like discriminative stimulus effects in rats (Wiley et al., 2004) and mice (unpublished data, JL Wiley et al.).

In addition to this behavioral evidence, both anandamide and WIN 55,212-2, but not THC, have been reported to stimulate [^{35}S]GTPγS binding in CB1 knockout mice (Breivogel, Griffin, Di Marzo, & Martin, 2001). Analysis of specific brain regions in these mice revealed significant [^{35}S]GTPγS binding increases in brain stem, cortex, hippocampus, diencephalon, midbrain, as well as in the spinal cord (Breivogel et al., 2001). Interestingly, [^{35}S]GTPγS binding was not enhanced in basal ganglia (composed of striatum and globus pallidus) or cerebellum tissue, providing evidence that localization of this putative novel cannabinoid receptor might not completely overlap with CB1 receptor distribution.

A recent study by Nguyen et al. (2010) examined agonist stimulated [^{35}S]GTPγS binding utilizing a novel 3D reconstruction of whole mouse brain. Results obtained in brains from CB1

knockout mice treated with WIN 55,212-2 largely echoed findings from Breivogel and colleagues (2001), including enhanced [^{35}S]GTPγS binding in brain stem, cortex, and hippocampus, with no significant difference noted in the basal ganglia (Nguyen et al., 2010). In contrast, increased binding was noticed in the cerebellum, potentially confined within the molecular layer (Nguyen et al., 2010). WIN 55,212-2 has also been shown to decrease the amplitude of excitatory postsynaptic currents in CB1 -/- and +/+ mice in the hippocampus, whereas inhibitory postsynaptic currents were only altered in CB1 +/+ mice (Hajos, Ledent, & Freund, 2001). These findings suggested the WIN 55,212-2 influences glutamatergic function through a non-CB1 mechanism.

As noted previously, the 3-substituent rimonabant analogs evaluated in this study produced tetrad activity in a similar manner as WIN 55,212-2 and O-6629's discriminative stimulus was partially generalized by WIN 55,212-2. The marked differences in the efficacy of these analogs and WIN 55,212-2 to further decrease body temperature and produce less catalepsy relative to THC might be suggestive of a shared non-CB1 mediated mechanism of action for these compounds. One methodological approach to test this hypothesis would be to determine whether cross-tolerance develops between WIN 55,212-2 and O-6629 in the tetrad following chronic administration of either drug.

While some similarities exist between the pharmacological profiles of WIN 55,212-2 and the 3-substituent rimonabant analogs evaluated in the present study, notable differences exist. WIN 55,212-2 has high affinity and efficacy at CB1 receptors, whereas O-6629 and O-6658 have moderate affinity for the receptor with no evidence of efficacy. Additionally, cross-generalization is observed between the discriminative stimulus effects of WIN 55,212-2 and THC (Wiley, 1999). In contrast, the rimonabant analogs did not produce THC-like

discriminative stimulus effects, nor did O-6629's discriminative stimulus generalize to THC. Nevertheless, the similar profiles of WIN 55,212-2 and the 3-substituent rimonabant analogs in the tetrad and partial substitution observed with WIN 55,212-2 in mice discriminating O-6629 combined with a number of non CB1-mediated effects observed with these compounds supports future testing to compare their pharmacological properties.

The fact that certain alterations in the 3-substituent of rimonabant conferred drastic functional changes in the pharmacodynamic and behavioral properties relative to the parent compound is quite interesting. Based upon findings obtained upon evaluation of O-6629 and O-6658 in the tetrad and drug discrimination, several avenues towards further elucidating the pharmacological properties of this class of 3-substituent rimonabant analogs are evident. First, generalization tests with other similar 3-substituent rimonabant analogs would help determine the pharmacological specificity of O-6629's discriminative stimulus and possible structural requisites for eliciting O-6629-like discriminative stimulus effects. O-6629 and O-6658 are nearly identical structurally, differentiated only by a bromine or azide group, respectively. It would be interesting to see if 3-substituent analogs with carbon chain substituents that produce cannabimimetic activity (Wiley et al., 2001) would engender O-6629's discriminative stimulus.

The similarities in magnitude of observed tetrad effects produced between these analogs and those reported for WIN 55,212-2 and WIN 55,212-2's partial substitution for O-6629 are also intriguing. Several lines of evidence strongly suggest that WIN 55,212-2 has activity at one or more novel cannabinoid receptors. One caveat to evaluating WIN 55,212-2 in O-6629 trained mice is that the high efficacy of WIN 55,212-2 at CB1 receptors might potentially mask the ability for the animal to detect non-CB1 mediated discriminative stimulus effects. To counteract this, WIN 55,212-2 could be given in the presence of rimonabant to block its activity at CB1

receptors. Another strategy to evaluate potential shared mechanism of action of O-6629 and WIN 55,212-2 would be to see if cross-tolerance develops to the effects of these compounds in the tetrad following chronic treatment with either.

Interestingly, 1 mg/kg nicotine also produced partial substitution (61.60 %DLR) for O-6629's discriminative stimulus. Full substitution for O-6629's discriminative stimulus was observed in 3 out of 5 subjects tested with this dose, whereas vehicle-appropriate responding was predominantly observed in the remaining two subjects. These differences in substitution patterns between subjects might be attributable to individual differences in stimulus control or sensitivity to a potential nicotinic acetylcholine component of O-6629's discriminative stimulus. Increasing the sample size for this generalization curve would be a prudent first step to help reduce the large margin of error present in the current sample. In the event that partial or even full substitution persisted in a larger number of subjects, challenge tests with the nicotinic antagonist mecamylamine against nicotine would provide insight as to whether nicotine's ability to engender O-6629-appropriate responding was due to nicotinic acetylcholine receptor activity. Similarly, a mecamylamine challenge against O-6629 would also provide evidence as to whether O-6629's discriminative stimulus was mediated through a nicotinic acetylcholine mechanism.

A number of findings have demonstrated functional interactions between nicotinic acetylcholine and cannabinergic systems. Rimonabant reduced nicotine self-administration behavior (Cohen, Perrault, Voltz, Steinberg, & Soubrie, 2002) and blocked cue-induced reinstatement of nicotine self-administration (Cohen, Perrault, Griebel, & Soubrie, 2005; De Vries, de Vries, Janssen, & Schoffelmeer, 2005), and reverse expression of nicotine conditioned place preference (Le Foll & Goldberg, 2004). In rats trained to discriminate THC from vehicle, nicotine and the muscarinic agonist pilocarpine both shifted the THC dose effect curve leftward,

but failed to substitute for THC when given alone (Solinas et al., 2007a). These shifts were reversed by nicotinic or muscarinic antagonists, respectively, but rimonabant only blocked the nicotine-induced shift. (Solinas et al., 2007a). Additionally, following pretreatment with the FAAH inhibitor URB597, nicotine produced high levels of THC-appropriate responding, which was partially reversed by rimonabant (Solinas et al., 2007a). Taken together with results of the present study, it is possible that nicotinic acetylcholine receptors might engage in crosstalk with both established and novel cannabinoid receptors. However, further investigation towards this end is necessary to test this hypothesis.

The prototypical opioid morphine failed to substitute for O-6629, but significant rate suppression was observed across all doses tested (1-10 mg/kg). Morphine and other opioids have frequently been evaluated in drug discrimination. An abundance of evidence points to μ-opioid mediation of morphine's discriminative stimulus, including cross-generalization between opiates such as fentanyl and morphine, lack of substitution with δ or κ-selective agonists, and blockade of discriminative stimulus effects with μ-opioid antagonists (for review, see Dykstra, Preston, & Bigelow, 1997). Thus, it is unlikely that O-6629 and other similar analogs produce their effects through μ-opioid receptors. This conclusion is not too surprising given the relative lack of antinociceptive efficacy of systemically administered O-6629 and O-6658 in the tetrad, even at doses that severely decreased spontaneous activity. It should be noted that maximal antinociception in the tail flick assay has been observed following intravenous administration with both of these analogs in ICR mice (JL Wiley, unpublished data).

Cocaine also did not engender O-6629's discriminative stimulus, with significant rate suppression observed with 10 and 30 mg/kg doses. Cocaine has been shown to exert discriminative control in laboratory animals through mesocorticolimbic dopaminergic activity

(Callahan, De La Garza, & Cunningham, 1997). While some degree of O-6629-appropriate responding was seen during generalization tests with cocaine, inspection of individual data revealed full substitution for O-6629 in one subject across all cocaine doses tested, with vehicle-like responding occurring in the majority of other subjects.

In sum, the results of the present study substantiated initial findings from our laboratory that suggested 3-substituent rimonabant analogs that produce cannabimimetic activity in the tetrad exert their behavioral effects through a non-CB1 mechanism. Analogs that retained a cyclic structure in the 3-substituent were highly unlikely to produce cannabimimetic activity, whereas certain carbon chain substitutions conferred agonist-like effects in the tetrad (JL Wiley unpublished data; present study). Notably, these effects were also present in CB1 receptor knockout mice and were not reversed by rimonabant pretreatment in wild-type mice (JL Wiley, unpublished data). O-6629 and O-6658, two novel 3-substituent rimonabant analogs evaluated in the present study, produced activity in the tetrad, but with noticeable differences in observed effect relative to THC. These compounds did not elicit THC-like discriminative stimulus effects in mice, nor did they modify the discriminative stimulus of the THC training dose. Finally, in mice discriminating O-6629 from vehicle, O-6658 dose-dependently substituted for the training drug. Additionally, partial substitution was observed with both WIN 55,212-2 and nicotine, whereas a number of other cannabinoid and non-cannabinoid test compounds failed to substitute for O-6629. Despite their affinity for CB1 receptors, the effects produced by these rimonabant analogs are almost certainly not attributable to CB1 receptor activity, nor do these compounds antagonize the classic pharmacological and discriminative stimulus effects of the prototypical cannabinoid THC.

Other worthwhile endeavors to pursue in mice trained to discriminate O-6629 would be to test anandamide in the presence of a FAAH inhibitor. Whether or not anandamide would substitute for O-6629 under more metabolically stable conditions is a matter of speculation, but anandamide has also been shown to produce cannabimimetic activity in the tetrad and other behavioral effects through a non-CB1, non-TRPV1 mechanism. The lack of any behavioral activity of anandamide (i.e., substitution or rate suppression) provides further justification for this proposed test. Generalization tests with chlorpromazine, a false positive in the tetrad, and diazepam, which produces partial substitution for THC's discriminative stimulus would also be appropriate choices.

One primary rationale for evaluating rimonabant analogs is to identify compounds that produce therapeutically beneficial effects with a lower incidence of side effects. Certainly, rimonabant's preclinical and clinical effects on appetite and its history as a marketed anti-obesity medication would suggest that some analogs might produce appetitive effects. Some preliminary evidence provided in this study demonstrated that a dose of O-6629 that did not alter locomotor activity markedly suppressed food-maintained operant response rates in a large percentage of mice tested in the THC discrimination experiment. However, limited inferences regarding these two findings can be made, given the increased demand on motor coordination to perform a specific operant response versus gross locomotor activity, coupled with the cognitive demand required of subjects in a drug discrimination task. Moreover, differences in motivational components for tests of spontaneous activity (no food reinforcement; free-feeding subjects) and drug discrimination (palatable food reinforcement; food-restricted subjects) also cloud interpretation. As mentioned previously, a series of studies investigating a range of doses of O-6629 and/or related analogs effects on locomotor activity and food-reinforced operant behavior

under several different schedules of reinforcement (e.g., fixed ratio and progressive ratio) would help delineate the role of the known motor suppressant effects produced by these analogs from potential appetitive and/or motivational influences.

Insofar as drug discrimination is one preclinical measure of a drug's abuse liability, generalization testing with morphine and cocaine would suggest that 3-substituent rimonabant analogs with similar pharmacological properties as O-6629 do not produce intoxicating effects similar to morphine or cocaine. Further testing with nicotine is necessary before determining the degree to which nicotine and O-6629 share discriminative stimulus effects before any clear inferences can be drawn in that regard.

Some of the most convincing neurochemical support for the existence of novel cannabinoid receptors is that WIN 55,212-2 stimulates [^{35}S]GTPγS binding in CB1 -/- mice. Thus, it would be interesting to see if O-6629 or other rimonabant analogs would be able to stimulate binding in CB1 -/- mice as well. In the event that enhanced binding was observed following drug treatment, regional distribution of receptor activation could provide insight into other physiological processes these compounds might influence.

In conclusion, the findings of the present study provide substantial behavioral evidence that the pharmacological properties of 3-substituent rimonabant analogs are not attributable to their affinity for CB1 receptors. In fact, based on the present findings demonstrating similarities between the tetrad profile of these analogs and WIN 55,212-2 and the observed partial substitution with the latter for O-6629, it is possible that these compounds are producing their effects, at least in part, through a novel cannabinergic mechanism. Further work is needed to substantiate this claim, but this possibility is certainly an intriguing one.

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