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The endogenous cannabinoid 2-arachidonoylglycerol is intravenously self-administered by squirrel monkeys

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Abstract

Two endogenous ligands for cannabinoid CB₁ receptors, anandamide and 2-arachidonoylglycerol (2-AG), have been identified and characterized. 2-AG is the most prevalent endogenous cannabinoid ligand in the brain and electrophysiological studies suggest 2-AG, rather than anandamide, is the true natural ligand for cannabinoid receptors and the key endocannabinoid involved in retrograde signaling in the brain. Here, we evaluated intravenously administered 2-AG for reinforcing effects in non-human primates. Squirrel monkeys that previously self-administered anandamide or nicotine under a fixed-ratio schedule with a 60-s timeout after each injection had their self-administration behavior extinguished by vehicle substitution and were then given the opportunity to self-administer 2-AG. Intravenous 2-AG was a very effective reinforcer of drug-taking behavior, maintaining higher numbers of self-administered injections per session and higher rates of responding than vehicle across a wide range of doses. To assess involvement of CB₁ receptors in the reinforcing effects of 2-AG, we pre-treated monkeys with the cannabinoid CB₁-receptor inverse agonist/antagonist rimonabant. Rimonabant produced persistent blockade of 2-AG self-administration without affecting responding maintained by food under similar conditions. Thus, 2-AG was actively self-administered by monkeys with or without a history of cannabinoid self-administration and 2-AG's reinforcing effects were mediated by CB₁ receptors. Self-administration of 2-AG by squirrel monkeys provides a valuable procedure for studying abuse liability of medications that interfere with 2-AG signaling within the brain and for investigating mechanisms involved in the reinforcing effects of endocannabinoids.

Introduction

Endocannabinoids are essential modulators of synaptic transmission throughout the central nervous system. Following release from postsynaptic neurons, endocannabinoids travel through the synaptic cleft to engage presynaptic cannabinoid CB₁ receptors. The outcome of this retrograde signaling is a decrease in synaptic transmission and short- or long-term synaptic modulation (Katona and Freund, 2008). 2-Arachidonoylglycerol (2-AG) was the second endogenous cannabinoid receptor ligand discovered after anandamide (Sugiura et al.,

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1995; Mechoulam et al., 1995), but anandamide has been far more extensively studied. Both 2-AG and anandamide are ligands for cannabinoid receptors (CB₁ and CB₂), but there are many differences between them. Levels of 2-AG in the brain are higher than those of anandamide and this difference appears similar in non-human primate and rodent brains (1000 fold versus 800 fold difference, respectively; Sugiura et al., 1995; Justinova et al., 2008), but lower differences (around 200 fold difference) in rodent brain were also reported (e.g., Bisogno et al., 1999). Although anandamide binds to cannabinoid receptors with a higher affinity than 2-AG, it acts only as a partial agonist (e.g., Mackie et al., 1993), while 2-AG acts as a full agonist at these receptors (Gonsiorek et al., 2000; Savinainen et al., 2001). Also, 2-AG selectively binds to cannabinoid receptors, while anandamide also interacts with non-cannabinoid binding sites such as vanilloid receptors (for review see Pertwee, 2008). Based on these differences between 2-AG and anandamide and on recent electrophysiology and anatomical studies, it has been proposed that 2-AG, rather than anandamide, is the true natural ligand for cannabinoid receptors and the key endocannabinoid involved in retrograde signaling in the brain (Sugiura et al., 2006; Katona and Freund, 2008; Tanimura et al., 2010).

Drugs of abuse affect central levels of 2-AG differently, depending on the abused drug, the brain area studied and the methodology employed. There is strong evidence for endocannabinoid involvement in the rewarding effects of Δ^9 -tetrahydrocannabinol (THC) (Di Marzo et al., 2000) and ethanol and opiates (Vigano et al., 2003; Caille et al., 2007), as opposed to psychostimulants such as cocaine (Gonzalez et al., 2002; Caille et al., 2007). Although, 2-AG is the most prevalent endogenous cannabinoid receptor ligand in the brain and appears to be involved in the rewarding effects of many abused drugs, its reinforcing effects have not yet been assessed in an animal model of drug abuse.

We previously demonstrated that anandamide, like THC, serves as an effective reinforcer of drug self-administration behavior in squirrel monkeys when injected intravenously (Justinova et al., 2005). Here, we studied reinforcing effects of intravenously self-administered 2-AG using the same squirrel monkey model. We used monkeys with a history of anandamide self-administration, as well as monkeys with a history of nicotine self-administration. We also evaluated whether cannabinoid CB₁ receptors mediated any reinforcing effects of 2-AG observed by pretreating monkeys responding for intravenous injections of 2-AG (and for comparison monkeys responding for THC or food under the same conditions) with the cannabinoid CB₁-receptor inverse agonist/antagonist rimonabant before experimental sessions.

Methods

Animals

Seventeen adult male squirrel monkeys (*Saimiri sciureus*) weighing 0.75 to 1.15 kg were housed in individual cages in a temperature- and humidity-controlled room with unrestricted access to water. Monkeys were fed (approximately two hours after the session) a daily ration of five biscuits of high protein monkey diet (Lab Diet 5045, PMI Nutrition International, Richmond, Indiana) and two pieces of Banana Softies (Bio-Serv, Frenchtown, NJ) that maintained body weights at a constant level throughout the study. Fresh fruits, vegetables and environmental enrichment were provided daily. Animals were maintained in facilities fully accredited by AAALAC and experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of the Intramural Research Program, NIDA, NIH, DHHS.

Two groups of four monkeys each were used for experiments with 2-AG: an anandamide group (70F4, 67F4, 42A, 6754) that had prior experience with self-administration of

anandamide and anandamide reuptake inhibitors (AM404 and VDM11) and a nicotine group (577, 431, 441, 572) that had prior experience with self-administration of nicotine. A third group of five animals that had experience with self-administration of THC was used to test rimonabant in monkeys (66B2, 453, 434, 25B, 37B). A fourth group of four monkeys was used to study the effects of rimonabant on responding reinforced by food (34A, 27B, 30A, 1549).

Apparatus

Experimental chambers and other apparatus used in this study were the same as previously described in detail (Justinova et al., 2003). Monkeys were surgically prepared with chronic indwelling venous catheters (polyvinyl chloride) and wore nylon-mesh jackets to protect the catheters. The monkey's catheter was connected to polyethylene tubing, which passed out of the isolation chamber where it was attached to a motor-driven syringe pump. Before the start of each session, monkeys were placed into the Plexiglas chairs and restrained in the seated position by waist locks.

Self-administration procedure

One-hour sessions were conducted five days per week (typically Monday to Friday). Before the start of each session, catheters were flushed with 1 ml of saline and one priming injection was delivered (calculated to fill the dead space of the IV catheter). At the start of the session, a white house light was turned off and green stimulus lights were turned on. In the presence of the green lights, monkeys were required to make 10 responses on a lever (10-response, fixed-ratio schedule of reinforcement; FR10) to produce an injection of anandamide. The completion of 10 responses on the lever turned off the green lights and produced an IV injection of 30 µg/kg of anandamide paired with a 2-s illumination of amber stimulus lights. Duration of each injection was 0.2 s and injection volume was 0.2 ml. Each injection was followed by a 60-s timeout period, during which the chamber was dark and lever presses had no programmed consequences. The same schedule and conditions were employed in groups of monkeys that had learned to respond for injections of nicotine (30 µg/kg) or THC (4 µg/kg) or for delivery of food (190 mg banana-flavoured food pellets; F0035, Bioserv, Frenchtown, NJ).

When responding for 30 µg/kg injections of anandamide or nicotine was stable for at least five consecutive sessions (less than 15% variability), self-administration behavior was extinguished by replacing anandamide or nicotine with its vehicle for five consecutive sessions, followed by replacement of vehicle with 3 µg/kg injections of 2-AG for five sessions. Then, in the anandamide-experienced monkeys, a 2-AG dose-effect curve was constructed by giving monkeys the opportunity to self-administer a range of 2-AG doses (0.1, 0.3, 1, 3, 10, 30, and 100 µg/kg/injection). Each 2-AG dose was studied for four to five consecutive sessions and each dose-condition was separated by four to five consecutive sessions of vehicle extinction. Afterwards we studied the effects of five-day pre-session treatment with 0.3 mg/kg rimonabant or its vehicle (given intramuscularly, i.m.; 60 minutes before session) on self-administration of the 3 µg/kg dose of 2-AG (a dose that maintained maximum rates of responding). Effects of vehicle or rimonabant (0.3 mg/kg) for five sessions were also determined in a group of monkeys responding for a 4 µg/kg THC injections (a dose of THC that maintains maximum rates of responding under these schedule conditions) and in a group of monkeys responding for food pellets under the same FR schedule of reinforcement. All monkeys were injected with vehicle or rimonabant in the chair and were then returned to their home cage until the start of the experimental session.

The monkeys used in the current study had extensive experience with vehicle extinction before the present experiments, including repeated episodes of extinction during initial

acquisition of baseline self-administration behavior. As a result, self-administration behavior ceased virtually immediately when vehicle is injected during the session in these monkeys. We have observed a gradual fall-off during the first several extinction periods during initial acquisition of anandamide, THC or nicotine self-administration behavior but this disappears with repeated extinction experience.

Data analysis

Cumulative-response records were obtained during all sessions in order to assess within-session patterns of responding. Reinforcements (injections or pellets) per session represent total number of injections delivered per session. Rates of responding are expressed as responses per second averaged over the one-hour session, with responding during time-outs not included in calculations. Data for dose-effect curves are expressed as mean response rates and numbers of reinforcements per session \pm SEM over the last three sessions. In addition, total intake of 2-AG for each dose was calculated. Effects of pretreatment with rimonabant on drug and food self-administration, as well as extinction and acquisition data, are expressed as mean \pm SEM of total numbers of reinforcements per session and rates of responding as a function of consecutive daily self-administration sessions. For statistical evaluation of effects of rimonabant or vehicle extinction over consecutive sessions, the last session of vehicle pretreatment was used as a control value to allow comparisons with subsequent sessions during pretreatment or extinction. For statistical evaluation of recovery of the behavior from extinction or rimonabant pretreatment over consecutive sessions, the last session of extinction or pretreatment was used as a control value to allow comparisons with subsequent sessions. Statistical analysis (SigmaStat program; Jandel Scientific, USA) was done using single-factor repeated measures ANOVA to assess differences between vehicle and test-drug pretreatment conditions or between different doses of 2-AG and vehicle. Significant main effects were analyzed further by subsequent paired comparisons to control values using Dunnett's test. Differences were considered statistically significant when $p < 0.05$.

Drugs

2-AG (2-arachidonoylglycerol) was provided by NIDA Research Resources Drug Supply Program (Bethesda, MD) and was dissolved in a vehicle containing 5% ethanol and 3% Tween 80 and sterile water to obtain stock solution of concentration 1.5 mg/ml, which was further diluted with sterile water as needed. Anandamide (N-Arachidonylethanolamine; synthesized in the laboratory of Dr. Alexandros Makriyannis at the Centre for Drug Discovery, Northeastern University, Boston, MA) and Δ^9 -tetrahydrocannabinol (THC; NIDA Research Resources Drug Supply Program, Bethesda, MD, USA) were dissolved in a vehicle containing 1% ethanol and 1% Tween 80 and saline. Rimonabant (SR141716; N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide), provided by NIDA Research Resources Drug Supply Program (Bethesda, MD), was dissolved in a vehicle of 2% ethanol, 2% Tween 80 and sterile water.

Results

Extinction of anandamide self-administration and acquisition of 2-AG self-administration

The monkeys learned to press a lever for intravenous injections of 30 μ g/kg of anandamide (FR10 schedule) during one-hour daily sessions. Injections of 30 μ g/kg anandamide maintained high response rates (mean of 0.68 ± 0.11 response per second) and high numbers of self-administered injections each session (mean of 47.08 ± 1.73 injections per session). When vehicle injections were substituted for anandamide injections, rates of responding ($F_{(5,15)} = 50.05$, $P < 0.001$), as well as number of injections self-administered per session ($F_{(5,15)} = 41.65$, $P < 0.001$), decreased dramatically (Figure 1A, B). When vehicle injections

were replaced with 3 µg/kg injections of 2-AG, self-administration behavior immediately recovered and, thus, acquisition of 2-AG self-administration was very rapid (Injections: $F_{(5,15)} = 27.06$, $P < 0.001$; Rates: $F_{(5,15)} = 5.20$, $P = 0.006$) (Figure 1A, B).

Extinction and reacquisition of 2-AG self-administration

During construction of a dose-response curve for 2-AG, vehicle extinction always preceded and followed testing of each dose of 2-AG. Substitution of vehicle for 2-AG (dose 30 µg/kg/injection showed) caused a significant decline in the number of injections delivered per session (Figure 1C; $F_{(4,12)} = 193.30$, $P < 0.001$) and in the rates of responding (Figure 1D; $F_{(4,12)} = 27.02$, $P < 0.001$). Replacement of vehicle injections with injections of 2-AG (dose 1 µg/kg/injection showed) resulted in immediate recovery of drug-taking behavior (Injections: $F_{(4,11)} = 18.17$, $P < 0.001$; Rates: $F_{(4,11)} = 5.31$, $P = 0.012$) (Figure 1C, D).

2-AG self-administration in nicotine-experienced monkeys

In a separate group of monkeys, we tested whether 2-AG is self-administered by monkeys without a history of cannabinoid self-administration. The monkeys learned to press a lever for intravenous injections of 30 µg/kg of nicotine (FR10 schedule) during one-hour daily sessions. Injections of 30 µg/kg nicotine maintained high response rates (mean of 1.46 ± 0.36 responses per second) and high numbers of self-administered injections each session (mean of 51.17 ± 3.17 injections per session). When vehicle was substituted for nicotine, rates of responding ($F_{(5,15)} = 15.54$, $P < 0.001$), as well as number of injections self-administered per session ($F_{(5,15)} = 112.76$, $P < 0.001$), decreased dramatically (Figure 2A, B). When vehicle injections were replaced with 3 µg/kg injections of 2-AG, self-administration behavior immediately recovered and 2-AG was self-administered by nicotine-experienced monkeys similarly to anandamide-experienced monkeys (Injections: $F_{(5,15)} = 41.97$, $P < 0.001$; Rates: $F_{(5,15)} = 8.00$, $P < 0.001$) (Figure 2A, B).

Self-administration of 2-AG

Varying the injection dose of 2-AG resulted in classic, inverted, U-shaped, dose-effect curves. 2-AG maintained significantly higher rates of responding ($F_{(7,21)} = 10.42$, $P < 0.001$; Figure 3B) and higher numbers of injections per session ($F_{(7,21)} = 28.25$, $P < 0.001$; Figure 3A) than vehicle at doses 1, 3, 10, and 30 µg/kg/injection. Maximal rate of responding (0.67 ± 0.09 response per second) and number of injections per session (46.75 ± 1.19 injections per session) were maintained by 3 µg/kg 2-AG per injection. The highest 2-AG intake per session was reached at a dose of 100 µg/kg/injection (2.91 ± 0.40 mg/session; Figure 3C).

Effects of cannabinoid CB₁ receptor blockade on responding maintained by 2-AG, THC and food pellets

Rimonabant at a dose of 0.3 mg/kg decreased the number of self-administered injections of 2-AG (Figure 4A; $F_{(5,10)} = 7.96$, $P = 0.003$) and THC (Figure 4B; $F_{(5,20)} = 34.66$, $P < 0.001$) to vehicle-control levels within a few sessions. Overall rates of responding were also significantly decreased during the five-day treatment with rimonabant (not shown; 2-AG: $F_{(5,10)} = 7.01$, $P = 0.005$; THC: $F_{(5,20)} = 35.25$, $P < 0.001$). 2-AG self-administration rapidly recovered when rimonabant treatment was stopped (2-AG: Injections: $F_{(3,6)} = 51.77$, $P < 0.001$; Rates: $F_{(3,6)} = 9.55$, $P = 0.011$; THC: Injections: $F_{(3,12)} = 26.70$, $P < 0.001$; Rates: $F_{(3,12)} = 13.50$, $P = 0.011$). Rimonabant at a dose 0.3 mg/kg did not alter responding maintained by food (Figure 4C: $F_{(3,15)} = 0.46$, $P = 0.80$, rates not shown: $F_{(3,15)} = 0.61$, $P = 0.69$).

Discussion

Using an intravenous drug self-administration model in squirrel monkeys, we found that 2-AG, like THC, cocaine and other abused drugs, was an effective reinforcer of drug-taking behavior. 2-AG was self-administered by monkeys that had a cannabinoid self-administration history (anandamide monkeys), as well as by monkeys with no history of cannabinoid self-administration (nicotine monkeys). The fact that 2-AG maintained drug-taking behavior in monkeys without a cannabinoid self-administration history suggests that reinforcing effects of 2-AG were not dependent on prior self-administration of a particular class of drugs. Rates of responding maintained by 2-AG injections were comparable to those maintained under the same conditions by THC, anandamide, methanandamide (a metabolism-resistant analog of anandamide) or cocaine injections in our previous studies (Tanda et al., 2000; Justinova et al., 2003; 2005; 2008).

Although rates of responding maintained by 2-AG injections were comparable to those maintained under the same conditions by other cannabinoid or non-cannabinoid drugs in our previous studies, 2-AG maintained very high levels of drug-taking behavior across a wider range of doses (1–30 µg/kg/injection) than is typical for other cannabinoid or non-cannabinoid drugs. For example, compared to a 30-fold difference between the highest and lowest dose in the effective dose range for 2-AG, there was only a fourfold difference between the highest and lowest dose in the effective dose range for THC (1–4 µg/kg/injection), anandamide (10–40 µg/kg/injection) and methanandamide (10–40 µg/kg/injection) in our previous self-administration studies (Tanda et al., 2000; Justinova et al., 2003; 2005). This may be explained by a combination of a very rapid metabolism of exogenously administered 2-AG in the brain and 2-AG's full efficacy at cannabinoid CB₁ receptors (Beltramo and Piomelli, 2000; Savinainen et al., 2001). It has been shown that a short duration of effects favors demonstration of a high frequency of self-administered injections under FR10 self-administration procedures (Ko et al., 2002). Thus, high potency and full efficacy at CB₁ receptors may allow 2-AG to function as a robust reinforcer at a very low dose (1 µg/kg/injection), while rapid degradation restricts accumulation of 2-AG in the brain with repeated injections over the session and, thus, delays the consequent drop in rates of responding and frequency of injection characteristic of the descending limb of the dose-response curve for most self-administered drugs and allows 2-AG to be an effective reinforcer at higher doses (up to 30 µg/kg/injection). Other cannabinoids acting as effective reinforcers of self-administration behavior do not exhibit this combination of pharmacokinetic and pharmacodynamic attributes. They are either rapidly metabolized in the brain, but have a lower efficacy (anandamide), or their metabolism is much slower than that of 2-AG (THC and methanandamide) (Agurell et al., 1986; Abadji et al., 1994).

The reinforcing effects of 2-AG in squirrel monkeys appear to be mediated by cannabinoid CB₁ receptors, since daily pre-session treatment with rimonabant resulted in complete blockade of 2-AG self-administration behavior. Rimonabant produced a similar complete blockade of THC self-administration behavior in the present study and has been shown to block reinforcing effects of THC, as well as anandamide and methanandamide, but not cocaine, in previous studies with squirrel monkeys under the same schedule conditions (Tanda et al., 2000; Justinova et al., 2005). The gradual fall-off in 2-AG self-administration during the five-day rimonabant regimen argues for antagonism of the 2-AG effects rather than a non-specific reduction in rates of responding. Moreover, in the present study, rimonabant did not depress responding for food under the same schedule conditions used with 2-AG, so it is unlikely that the effects of rimonabant on 2-AG self-administration behavior were due to non-specific depressant effects.

Anandamide and 2-AG are implicated in the pathophysiology of pain and anxiety, as well as many neuropsychiatric disorders, including Alzheimer's disease, multiple sclerosis and addiction. It is therefore understandable that there is a wide interest in developing medications that promote the actions of both anandamide and/or 2-AG throughout the brain, e.g., by inhibiting their membrane transport or their metabolism (see reviews by Piomelli, 2005; Petrosino and Di Marzo, 2010). Since endogenously released cannabinoids can activate brain reward processes via CB₁ receptors, which could lead to unwanted psychological side effects and eventually to drug abuse and addiction, there has been concern about abuse liability of potential therapeutics that enhance actions of endogenously released endocannabinoids, such as inhibitors of the primary anandamide-degrading enzyme fatty acid amide hydrolase (FAAH). Recent experiments in squirrel monkeys and rats have failed to find abuse-related rewarding or relapse-inducing effects with the most frequently studied FAAH inhibitor URB597 (Justinova et al., 2008.; Scherma et al., 2008), but newer FAAH inhibitors remain to be studied. The questions regarding the abuse liability of FAAH inhibitors are now being asked about recently developed inhibitors of the primary 2-AG degrading enzyme, monoacylglycerol lipase (MAGL; Dinh et al., 2002; Minkkila et al., 2010). Emerging evidence from rodent studies is showing that there are fundamental differences in the mode of signaling for the anandamide and 2-AG signaling pathways (Schlosburg et al., 2010), but also that these two major endocannabinoid pathways engage in extensive interactions in the nervous system (Long et al., 2009). However, to date there are no published reports on reinforcing/rewarding effects of drugs that selectively increase 2-AG levels in the brain, like MAGL inhibitors, or dual FAAH/MAGL inhibitors, which increase levels of both 2-AG and anandamide.

The present study shows that the endocannabinoid 2-AG functions as a positive reinforcer in monkeys with or without a cannabinoid self-administration history and that its reinforcing effects are mediated by cannabinoid CB₁ receptors. Thus, intravenous self-administration of 2-AG by squirrel monkeys provides a valuable procedure for studying abuse liability of medications that interfere with brain 2-AG signaling and for investigating mechanisms involved in the reinforcing effects of endocannabinoids.

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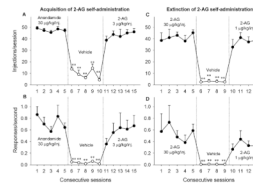


Figure 1.

Acquisition, extinction and reacquisition of 2-AG self-administration behavior over consecutive sessions. Acquisition: Number of injections per session (A) and overall rates of responding (B) in the presence of a green light during 30 µg/kg/injection anandamide sessions (sessions 1–5), vehicle sessions (sessions 6–10), and 2-AG 3 µg/kg/injection sessions (sessions 11–15) are shown. Extinction and reacquisition: Number of injections per session (C) and overall rates of responding (D) during 30 µg/kg/injection 2-AG sessions (sessions 1–5), vehicle sessions (sessions 6–9), and 2-AG 1 µg/kg/injection sessions (sessions 10–13) are shown. Points represent means ± SEM from four subjects. ** $p < 0.01$, post-hoc comparisons with the last session prior to vehicle extinction (session 5).

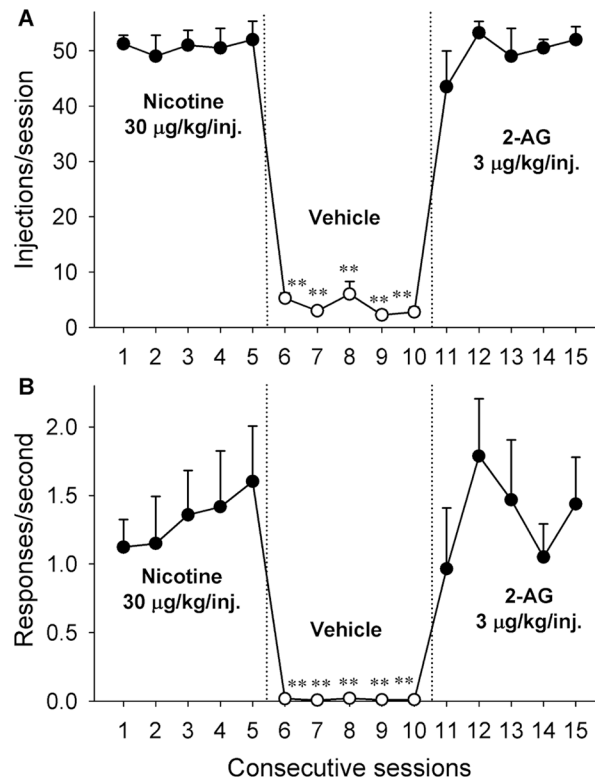


Figure 2.

2-AG self-administration in nicotine-experienced monkeys over consecutive sessions. Number of injections per session (A) and overall rates of responding (B) in the presence of a green light during 30 µg/kg/injection nicotine sessions (sessions 1–5), vehicle sessions (sessions 6–10), and 2-AG 3 µg/kg/injection sessions (sessions 11–15) are shown. Points represent means \pm SEM from four subjects. $**p < 0.01$, post-hoc comparisons with the last session prior to vehicle extinction (session 5).

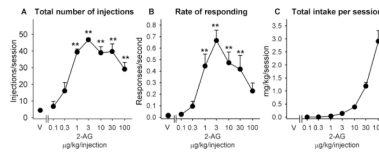
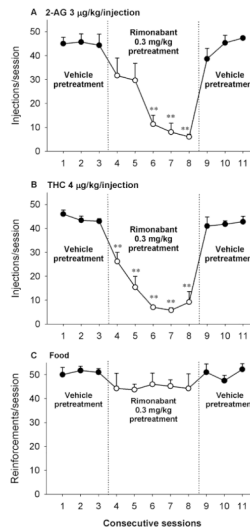


Figure 3. Effects of varying injection dose on self-administration of 2-AG. Number of injections of 2-AG per session (A), overall rates of responding in the presence of the green light signaling 2-AG availability (B) and total 2-AG intake per session (C) are shown as a function of injection dose of drug. Each point represents the mean \pm SEM ($n = 4$) of the last three sessions under each 2-AG unit dose condition and under a vehicle condition. $**p < 0.01$ post-hoc comparisons with the vehicle conditions. Symbol “V” represents vehicle.

**Figure 4.**

Effects of rimonabant given as a pretreatment before experimental session on responding maintained by 2-AG (A), THC (B) or food (C), during five consecutive sessions. Numbers of injections per session after i.m. pretreatment with vehicle (sessions 1–3 and 9–11) or 0.3 mg/kg rimonabant (sessions 4–8) are shown. Each point represents the mean \pm SEM from three (A), five (B) or four (C) monkeys. * $p < 0.05$, ** $p < 0.01$, post-hoc comparisons with the last session with vehicle pretreatment (session 3) prior to pretreatment with rimonabant.