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RESEARCH PAPER

Inhibition of monoacylglycerol lipase reduces nicotine withdrawal

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BACKGROUND AND PURPOSE

Abrupt discontinuation of nicotine, the main psychoactive component in tobacco, induces a withdrawal syndrome in nicotine-dependent animals, consisting of somatic and affective signs, avoidance of which contributes to drug maintenance. While blockade of fatty acid amide hydrolase, the primary catabolic enzyme of the endocannabinoid arachidonoylethanolamine (anandamide), exacerbates withdrawal responses in nicotine-dependent mice, the role of monoacylglycerol lipase (MAGL), the main hydrolytic enzyme of a second endocannabinoid 2-arachidonylglycerol (2-AG), in nicotine withdrawal remains unexplored.

EXPERIMENTAL APPROACH

To evaluate the role of MAGL enzyme inhibition in nicotine withdrawal, we initially performed a genetic correlation approach using the BXD recombinant inbred mouse panel. We then assessed nicotine withdrawal intensity in the mouse after treatment with the selective MAGL inhibitor, JZL184, and after genetic deletion of the enzyme. Lastly, we assessed the association between genotypes and smoking withdrawal phenotypes in two human data sets.

KEY RESULTS

BXD mice displayed significant positive correlations between basal MAGL mRNA expression and nicotine withdrawal responses, consistent with the idea that increased 2-AG brain levels may attenuate withdrawal responses. Strikingly, the MAGL inhibitor, JZL184, dose-dependently reduced somatic and aversive withdrawal signs, which was blocked by rimonabant, indicating a CB_1 receptor-dependent mechanism. MAGL-knockout mice also showed attenuated nicotine withdrawal. Lastly, genetic analyses in humans revealed associations of the *MAGL* gene with smoking withdrawal in humans.

CONCLUSIONS AND IMPLICATIONS

Overall, our findings suggest that MAGL inhibition maybe a promising target for treatment of nicotine dependence.

Abbreviations

2-AG, 2-arachidonylglycerol; AEA, arachidonoylethanolamine (anandamide); AMG, amygdala (AMG); FAAH, fatty acid amide hydrolase; Hip, hippocampus (Hip); KO, knockout; MAGL, monoacylglycerol lipase; NAc, nucleus accumbens (NAc); PFC, prefrontal cortex; THC, Δ^9-tetrahydrocannabinol; VTA, ventral tegmental area; WT, wild type

Tables of Links

TARGETS
Enzymes[a]
MAGL, monoacylglycerol lipase
FAAH, fatty acid amide hydrolase
GPCR[b]**s**
CB_1 receptors
CB_2 receptors

LIGANDS
2-AG, 2-arachidonylglycerol
AEA, anandamide
CP55940
JZL184
Mecamylamine
Nicotine
Rimonabant, SR141716A

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Tobacco is one of the most widely abused drugs and the leading cause of preventable death worldwide (World Health Organization, 2012). Nicotine, the main psychoactive component in tobacco, is essential in the initiation and maintenance of tobacco addiction. Cessation of smoking elicits a withdrawal syndrome characterized by both physical and affective withdrawal symptoms (Stolerman and Shoaib, 1991; American Psychiatric Association, 1994; Mendrek *et al.*, 2006). Rodents also manifest these signs after cessation of chronic nicotine administration and exhibit symptoms consistent with those reported in humans (Damaj *et al.*, 2003; De Biasi and Dani, 2011). Although the mechanisms underlying the manifestation of nicotine withdrawal symptoms are not clearly defined, emerging evidence suggests a possible role for the endocannabinoid system. The best-characterized endocannabinoids include *N*-arachidonoylethanolamine (anandamide; AEA) (Devane *et al.*, 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995), which produce their physiological effects primarily by activating cannabinoid CB_1 and CB_2 receptors (Matsuda *et al.*, 1990; Munro *et al.*, 1993; Cabral and Marciano-Cabral, 2005). The levels of AEA and 2-AG are tightly regulated by their main synthetic and degradative enzymes. For example, AEA and 2-AG are degraded by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (Cravatt *et al.*, 1996; Dinh *et al.*, 2002). The development of pharmacological and genetic strategies to prolong the availability of AEA and 2-AG by inactivating FAAH and MAGL, respectively, has advanced our understanding of the role that endocannabinoids play in many physiological processes (Castañé *et al.*, 2005; Merritt *et al.*, 2008). Furthermore, recent work has highlighted the relevance of the endocannabinoid system in the regulation of nicotine reward and dependence. It has been demonstrated that administration of Δ^9-tetrahydrocannabinol (THC), the primary active constituent of marijuana, decreases somatic withdrawal signs in nicotine-dependent mice through a CB_1 receptor-mediated mechanism (Balerio *et al.*, 2004), suggesting that CB_1 agonists might alleviate nicotine withdrawal symptoms. However, exogenous administration of CB_1 receptor agonists produces untoward side effects (Lichtman and Martin, 1991; Martin *et al.*, 1999; Darmani, 2001). Recent studies suggest that inhibitors of FAAH or MAGL might have the therapeutic utility of exogenous CB_1 receptor agonists, such as THC, without producing as many undesirable side effects (Cravatt *et al.*, 1996; Solinas *et al.*, 2007; Ahn *et al.*, 2008; 2009; Justinova *et al.*, 2008; Long *et al.*, 2009a,b). We previously reported that FAAH inhibition exacerbates withdrawal responses of nicotine-dependent mice (Merritt *et al.*, 2008; Muldoon *et al.*, 2013). However, the role of 2-AG in nicotine dependence has not been addressed. 2-AG is approximately 200-fold more abundant than AEA in the brain, acts as a full agonist at both CB_1 and CB_2 receptors (Gonsiorek *et al.*, 2000; Savinainen *et al.*, 2001), and is regarded as the chief endocannabinoid involved in retrograde signalling in the nervous system (Pan *et al.*, 2009). In this study, we investigated the functional contribution of MAGL to a critical component of nicotine addiction, the manifestations of the nicotine withdrawal syndrome. For this purpose, both behavioural and genetic techniques in rodents and in humans were used to establish the role of MAGL in nicotine withdrawal.

Methods

Animals

All animal care and experimental procedures complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A N of 6–8 animals were used for each experimental group.

Naive male 8- to 10-week-old ICR mice (Harlan Laboratories, Indianapolis, IN, USA) and male and female (equal number) MAGL knockout (KO) and MAGL wild-type (WT) mice on a mixed 129SvEv/C57BL/6J background from the Scripps Research Institute, La Jolla, CA (for more information regarding initial breeders, see Schlosburg *et al.*, 2010) were

used. Mice were housed five per cage in a 21°C humidity-controlled facility with *ad libitum* access to food and water. The animal facility was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were performed during the light cycle.

Genetic correlation analyses using the BXD recombinant inbred panel

Mecamylamine-induced somatic signs of withdrawal following chronic nicotine administration across the BXD recombinant inbred mouse panel have been previously phenotyped by our laboratory (Jackson *et al.*, 2011). BXD lines were derived by crossing C57BL/6J and DBA/2J and inbreeding progeny for 20 or more generations. Somatic signs were scored as described in the Methods section. Using the GeneNetwork web resource for the study of genetics and genomics in complex traits (http://www.GeneNetwork.org), mecamylamine-induced somatic signs of nicotine withdrawal were screened for strong correlations with basal MAGL mRNA expression in whole brain ($n = 26$ lines and $n = 8$ mice per line per treatment) (UCHSC BXD Whole Brain mRNA M430 2.0, Nov 2006 RMA Database) and forebrain/midbrain ($n = 23$) (INIA BXD Brain mRNA M430, June 2006 RMA Database) expression databases. All comparisons reported are Pearson's product-moment correlations.

Withdrawal studies

Nicotine chronic administration protocol. Mice were anaesthetized with sodium pentobarbital (45 mg·kg^{-1}, i.p.) and implanted s.c. with Alzet osmotic minipumps (model 2002; Durect Corporation, Cupertino, CA, USA). The concentration of nicotine was adjusted according to animal weight and the minipump flow rate was also adjusted to deliver 24 mg·kg^{-1}·day^{-1} for 14 days.

Precipitated nicotine withdrawal studies. The effects of the MAGL inhibitor, JZL184, on nicotine withdrawal were assessed as previously described (Malin *et al.*, 1994; Tizabi *et al.*, 1997; Jackson *et al.*, 2008). To evaluate the acute effects of JZL184 administration on precipitated withdrawal, mice were infused with nicotine (24 mg·kg^{-1}·day^{-1}) or saline for 14 days. On day 15, the subjects were given an i.p. injection of either vehicle (i.p.) or JZL184 (4, 8 and 40 mg·kg^{-1}, i.p.), followed by mecamylamine injection (2 mg·kg^{-1}, s.c.) at 120 min, and at 130 min, mice were observed for withdrawal signs during a 20 min observation period. Withdrawal signs measured included paw and body tremors, head shakes, backing, jumps, curls and ptosis. These signs were then averaged together to obtain the average somatic sign score (±SEM).

A separate experiment was conducted by administering the lowest effective dose of JZL184 (8 mg·kg^{-1}, i.p.) in combination with the CB_1 antagonist rimonabant in order to determine if the effect of JZL184 was CB_1 receptor mediated. Mice were treated with rimonabant (0.3 and 3 mg·kg^{-1}, i.p.) 120 min after JZL184 administration. After 30 min, mice were treated with mecamylamine (2 mg·kg^{-1}, s.c.) and tested 10 min later. Mice were observed for somatic withdrawal signs for 20 min as described previously. Lastly, we assessed the effect of genetic deletion of MAGL on nicotine withdrawal. MAGL WT and KO mice were treated with nicotine to induce dependence as described earlier, then received mecamylamine (2 mg·kg^{-1}, s.c.), and 10 min later, mice were observed for a 20 min period to measure somatic withdrawal signs. These signs were then averaged together to obtain the average somatic sign score (±SEM).

Spontaneous nicotine withdrawal study. To assess the effects of acute JZL184 administration on spontaneous withdrawal from nicotine, mice were infused with nicotine or saline for 14 days. On day 14, mice were anaesthetized under isoflurane anaesthesia and minipumps were removed in the evening. On day 15, 18–24 h after minipump removal, these mice were pretreated for 2 h with either vehicle (i.p.) or JZL184 (2, 4 and 8 mg·kg^{-1}, i.p.) and were observed for somatic withdrawal signs for 20 min as described earlier. Guided by our results from the precipitated nicotine withdrawal, we started our spontaneous withdrawal with the lowest effective dose of JZL184 (8 mg·kg^{-1}). Since we saw an effect at this dose, we continued to lower doses until we demonstrated a loss of effect of JZL184 on nicotine withdrawal. All studies were performed by an observer unaware of the experimental treatment. In addition, the doses of JZL184 used in our studies had no significant effects on the locomotor activity of mice (Kinsey *et al.*, 2011).

Repeated administration of JZL184 on precipitated withdrawal studies. Mice were implanted with either nicotine or saline minipumps for 14 days. On day 9 of minipump infusion, the mice were divided into groups. The first group received JZL184 (8 mg·kg^{-1}, i.p.) once a day for 5 days, whereas the second group received vehicle (i.p.) for 5 days. On day 15 (test day), these mice were further divided into groups in which repeated JZL184 administered animals were given either vehicle (i.p.) or JZL184 (8 mg·kg^{-1}, i.p.). Furthermore, repeated vehicle administered animals were given either JZL184 (8 mg·kg^{-1}, i.p.) or vehicle (i.p.) 2 h prior to administration of mecamylamine (2 mg·kg^{-1}, s.c.) or vehicle (s.c.) and withdrawal signs were evaluated as described earlier. Immediately after withdrawal testing, the nucleus accumbens (NAc), amygdala (AMG) and ventral tegmental area (VTA) were dissected using the Paxinos and Franklin (54) as a guide. The samples were then frozen in liquid nitrogen and then stored at –80°C until CP55940-stimulated [^{35}S] GTPγS and [^{3}H] SR141716A binding assays were conducted.

Conditioned Place Aversion (CPA)

The CPA protocol was conducted over the course of 4 days in a biased fashion as described in Jackson *et al.* (2008). Briefly, mice were implanted with 28 day nicotine (36 mg·kg^{-1}·day^{-1}) or saline minipumps 14 days prior to initiation of CPA testing to induce dependence. Infusion continued throughout the duration of testing. On day 1 of the CPA procedure, after a 5 min habituation period in the centre compartment, mice were allowed to roam freely between compartments for 15 min to determine baseline responses. The pre-preference score was used to pair each mouse with mecamylamine (3.5 mg·kg^{-1}, s.c.) to its initially preferred compartment. On days 2 and 3 of CPA training, all mice received injections of saline in the morning and mecamylamine in the afternoon.

In addition, 2 h prior to afternoon conditioning, animals received JZL184 (8 mg·kg^{-1}, i.p.) or vehicle (i.p.). In a separate experiment, before afternoon conditioning, rimonabant (3 mg·kg^{-1}, i.p.) was administered 30 min after JZL184 administration. After 30 min, mice were treated with mecamylamine (2 mg·kg^{-1}, s.c.). On day 4 in the afternoon, mice moved freely between compartments same as day 1 and time spent in each compartment was recorded for each mouse. A reduction in time spent in the initially preferred compartment was interpreted as CPA.

Extraction and quantification of brain EC levels with LC-MS

ICR male mice were injected with either JZL184 (8 mg·kg^{-1}, i.p.) or vehicle (i.p.), and 2 h later, the prefrontal cortex (PFC), NAc, hippocampus (Hip), AMG and VTA were dissected using the Paxinos and Franklin (2012) as a guide. Brain regions from each mouse were immediately frozen in liquid nitrogen and stored at –80°C until the time of processing.

On the day of processing, tissues were weighed and homogenized with 1.4 mL of chloroform–methanol (2:1, v/v; containing PMSF (0.0348 mg mL^{-1}) after the addition of internal standards to each sample (2 pmol of AEA-d8, 1 nmol of 2-AG-d8 and 1 nmol AA-d8). Homogenates were then mixed with 0.3 mL of 0.73% (w/v) NaCl, vortexed and then centrifuged for 10 min at 3220× *g* at 4°C. The aqueous phase was collected and extracted two more times with 0.8 mL of chloroform. The organic phases from the three extractions were pooled, and the organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 mL of chloroform and mixed with 1 mL of ice-cold acetone. The mixtures were then centrifuged for 5 min at 1811× *g* at 4°C. The upper layer of each mixture was collected and evaporated under nitrogen. Dried samples were reconstituted with 0.1 mL of methanol and placed in autosample vials for analysis.

Liquid chromatography-tandem mass spectrometry was used to quantify AEA, 2-AG and arachidonic acid (AA). The mobile phase consisted of water–methanol (10:90) with 0.1% ammonium acetate and 0.1% formic acid. The column used was a Discovery HS C18, 4.6 × 15 cm, 3 µm column (Supelco, Bellefonte, PA, USA). Ions were analysed in multiple reaction monitoring mode and the following transitions were monitored in positive mode: (348 > 62) and (348 > 91) for AEA; (356 > 62) for AEA-d8; (379 > 287) and (279 > 269) for 2-AG; (387 > 96) for 2AG-d8; in negative mode: (303 > 259) and (303 > 59) for AA and (311 > 267) for AA-d8. A calibration curve was constructed for each assay on the basis of linear regression using the peak area ratios of the calibrators. The extracted standard curves ranged from 0.039 to 40 pmol for AEA, from 0.0625 to 64 nmol for 2-AG and from 1 to 32 nmol for AA.

CP55, 940-stimulated [^{35}S]GTPγS binding in membranes

Each sample was homogenized in an ULTRA-TURRAX T25 in cold membrane buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4) and centrifuged at 48 000× *g* for 10 min at 4°C. Pellets were re-suspended in membrane buffer and centrifuged again at 48 000× *g* for another 10 min at 4°C. Pellets from the second centrifugation were homogenized in membrane buffer to measure protein concentration and then incubated for 10 min at 30°C in 0.004 U·mL^{-1} adenosine deaminase. Concentration–effect curves were generated by incubating appropriate amounts of membrane protein (5–10 µg) in assay buffer with 0.10 nM [^{35}S]GTPγS, 30 µM GDP, 0.1% BSA and, 1–300 nM CP55,940 in a final volume of 0.5 mL at 30°C for 2 h. Basal binding was determined in the absence of agonist and non-specific binding was measured in the presence of 30 µM GTPγS. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B filters, followed by three washes with cold 50 mM Tris–HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 50% efficiency for ^{35}S after 1 h shaking of the filters in 4 mL of Budget Solve scintillation fluid (Sim-Selley *et al.*, 2006).

[^{3}H]SR141716A binding in membranes

Membranes were prepared as described previously and appropriate concentrations (X-Y µg) were incubated for 90 min at 30°C in buffer A (50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA and 100 mM NaCl, pH 7.4) with 0. 5% BSA and varying concentrations of [^{3}H]SR141716A in a total volume of 0.5 mL. Non-specific binding was assessed in the presence of 2 µM unlabelled SR141716A. Incubations were terminated by vacuum filtration through Whatman GF/B filters and washed three times with ice-cold 50 mM Tris buffer with 0.25% BSA Tris–HCl, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for ^{3}H after extraction of the filters in scintillation fluid (Sim-Selley *et al.*, 2006).

Human genetic association analyses

Genetic association analyses of the *MAGL* gene were conducted with the study of addiction with gene and environment (SAGE) and genetic architecture of smoking and smoking cessation data sets (SC). Data from three questions of the Fagerstrom Test for Nicotine Dependence (FTND) were used to correlate with data from mouse withdrawal studies: (i) How soon after you wake up do you smoke your first cigarette (within 5, 6–30, 31–60 or after 60 min, FTND1)? (ii) Which cigarette you hate most to give up (the first one in the morning or any other FTND2)? and (iii) Do you find it difficult to refrain from smoking in places where it is forbidden (yes or no FTND3)? These three questions were the only questions from the FTND questionnaire that are directly relevant to nicotine withdrawal. FTND1 data were categorical, and we treated them as quantitative trait in our analyses. FTND2 and FTNND3 were binary data and were analysed by logistic regression. For both data sets, we used both African-American and European-American subjects. There were 2887 European-American and 1420 African-American subjects in the SAGE sample, and there were 2483 European-Americans and 727 African-American subjects in the SC sample. We selected all SNPs in a 200 kB interval (hg19, Chr3:127 375 000–127 575 000) containing the *MAGL* gene for both data sets, tested the association between genotypes and the smoking withdrawal phenotypes. In the SAGE data set, there were 51 genotyped SNPs and there were 45 SNPs for the SC data set in this interval. We first conducted association

analyses using linear (FTND1) or logistic (FTND2 and FTND3) regression for each ethnic group and then combined the results by meta-analysis. Because we combined data from two ethnic groups, we used the random effect model in the GWAMA program and examined the heterogeneity between the African-American and European-American groups. As we tested 51 SNPs, the *P*-value threshold for significance was 0.00098.

Data analysis

Statistical analyses of all behavioural data were performed using StatView® (SAS Institute, Cary, NC, USA). All behavioural scores were expressed as mean ± SEM. for each group. Statistical analyses of all studies were analysed using either one-way or two-way ANOVA, where appropriate, with treatment as the between-subject factor. *P*-values < 0.05 were considered to be statistically significant. Significant results were further analysed using the Student–Newman–Keuls *post hoc* test.

Materials

(–)-Nicotine hydrogen tartrate salt and mecamylamine hydrochloride were purchased from Sigma-Aldrich (St Louis, MO, USA). The CB_1 receptor antagonist rimonabant [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl] was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD, USA). JZL184 was synthesized as described previously (Long *et al.*, 2009a) by Organix, Inc. (Woburn, MA, USA). JZL184 and rimonabant were dissolved in ethanol, followed by addition of Emulphor-620 (Rhone-Poulenc, Princeton, NJ, USA) and diluted with 0.9% saline to form a vehicle mixture of ethanol–emulphor–saline in a ratio of 1:1:18. Nicotine and mecamylamine were dissolved in physiological saline (0.9% sodium chloride). All injections were administered in a volume of 10 mL·kg^{-1}. Nicotine and mecamylamine were administered via s.c. injection, whereas JZL184 and rimonabant were given via i.p. injection. All doses are expressed as the free base of the drug. GTPγS, adenosine deaminase and BSA were purchased from Sigma-Aldrich. $^{[35S]}$GTPγS (1250 Ci·mmol^{-1}) and $^{[3H]}$SR141716A (44.0 Ci·mmol^{-1}) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Budget Solve (scintillation fluid) was purchased from Research Products Int. Corp. (Mount Prospect, IL, USA) and Whatman GF/B glass fibre filters were obtained from Brandel (Gaithersburg, MD, USA).

Results

MAGL mRNA expression in brain significantly correlates with somatic signs of nicotine withdrawal

As an initial step to investigate the possible role of 2-AG in nicotine withdrawal, we performed genetic correlation analyses between nicotine withdrawal phenotypes of the BXD recombinant inbred panel of mice previously generated by our laboratory using a similar nicotine withdrawal protocol (Jackson *et al.*, 2011) and expression levels of MAGL in the brain. Mecamylamine-induced somatic withdrawal signs following chronic nicotine (GeneNetwork Trait ID 15572) showed significant positive correlations with basal mRNA levels of the *MAGL* gene (Mgll; probe set 1426785_s_at) in both whole brain (Figure 1A) and forebrain/midbrain (Figure 1B) across the BXD recombinant inbred strains (A, Pearson's r = 0.442, P = 2.30E-2; B, Pearson's r = 0.430, P = 3.99E-2). These findings are consistent with the premise that decreased 2-AG signalling may contribute to the severity of somatic symptoms of nicotine withdrawal.

Inhibition or genetic deletion of MAGL attenuates precipitated somatic withdrawal signs in nicotine-dependent mice

We next tested the effects of the MAGL inhibitor JZL184 on somatic signs in nicotine-treated mice undergoing precipitated withdrawal. As shown in Figure 2, nicotine-dependent

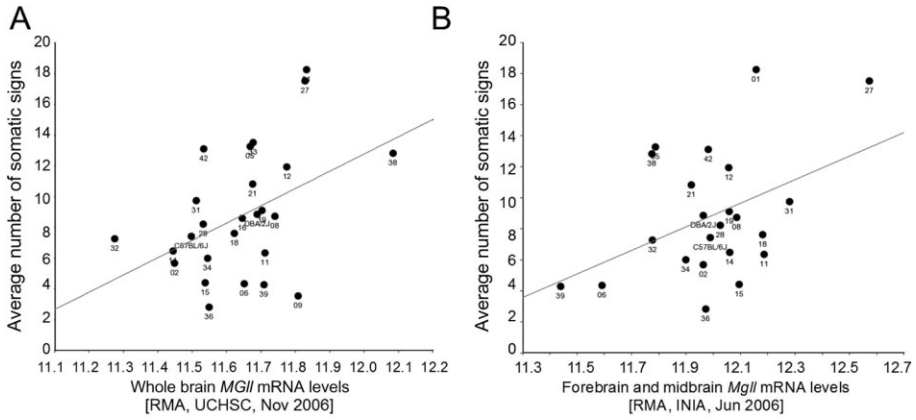

Figure 1

Significant positive correlations between basal MAGL mRNA transcript levels (probe set 1426785_s_at) in whole brain (A) and forebrain/midbrain (B) with mecamylamine-induced somatic signs following chronic nicotine administration (GeneNetwork Trait ID 15572). $P < 0.05$.

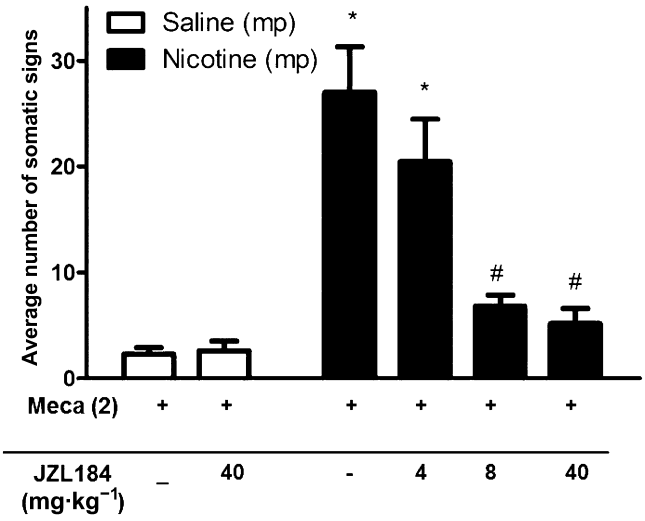

Figure 2

Acute MAGL inhibition by JZL184 (4, 8 and 40 mg·kg^{-1}, i.p.) dose-dependently attenuated precipitated nicotine somatic withdrawal signs. JZL184 was administered 2 h before mecamylamine (Meca (2); 2 mg·kg^{-1}, s.c.) treatment. The total withdrawal signs measure consists of paw tremors, head shakes, backing, ptosis and jumping. Data are expressed as mean ± SEM of n = 6–8 mice per group. *P < 0.05 versus all treatment groups; #P < 0.05 versus nicotine (mp) + Meca (2) group and nicotine (mp) + JZL184 (4) + Meca(2). mp = minipump.

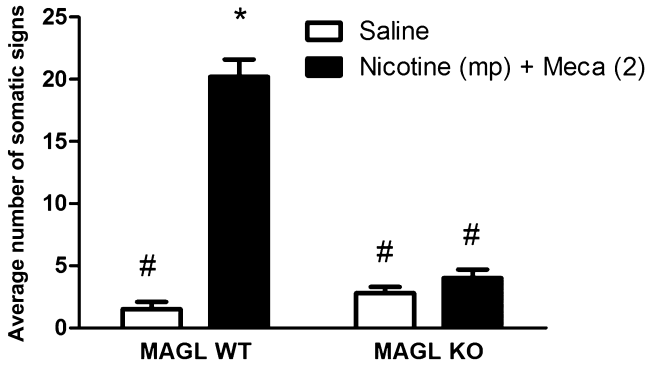

Figure 3

Genetic deletion of MAGL prevented precipitated nicotine somatic withdrawal signs. MAGL KO and WT mice implanted with 14 day minipumps were chronically infused with either nicotine (24 mg·kg^{-1}) or saline. On the day of withdrawal, mice were administered mecamylamine (Meca (2); 2 mg·kg^{-1}, s.c) and somatic signs were observed. MAGL KO mice exhibited reduced nicotine somatic withdrawal signs compared to their WT counterparts. Data are expressed as mean ± SEM of n = 6 mice per group. *P < 0.05 versus all treatment groups; #P < 0.05 versus nicotine (mp) + Meca (2) wild type. mp = minipump.

mice given mecamylamine (2 mg·kg^{-1}, s.c.) displayed significant increases in the incidence of somatic withdrawal signs [F(4, 37) = 20.55; P < 0.0001].

Pretreatment with JZL184 (4, 8 and 40 mg·kg^{-1}, i.p.) dose-dependently decreased the expression of precipitated nicotine somatic withdrawal signs. A significant interaction between JZL184 and mecamylamine treatment was found [F(3, 45) = 6.0; P < 0.002] (Figure 2). Finally, none of the doses of JZL184 or mecamylamine used in this assessment significantly affected responses in saline-infused mice [F (4, 30) = 1.4; P < 0.3] (Supporting Information Fig. S1).

To assess the extent to which a behaviourally active dose of JZL184 altered ECs, the levels of 2-AG and AEA, as well as AA, were measured in PFC, NAc, HIP, AMG and VTA that were collected 2 h after administration of JZL184 (8 mg·kg^{-1}, i.p.) or vehicle. The effect of JZL184 (8 mg·kg^{-1}) on endocannabinoid levels in the various brain regions is shown in Table 1A. JZL184 significantly increased 2-AG levels by 4- to 10-fold compared with vehicle in the PFC [F(1, 8) = 20.8; P < 0.01], Hip [F(1, 8) = 6.1; P < 0.05], AMG [F(1, 8) = 46.7; P < 0.0001], VTA [F(1, 8) = 8.4; P < 0.05] and NAc [F(1, 8) = 18.4; P < 0.01]. In contrast, this dose of JZL184 did not significantly affect AEA or AA levels (Table 1B and C) in any of the brain regions examined.

The effect of 2-AG on somatic signs of nicotine withdrawal was further evaluated in mice with genetic deletion of MAGL. Examination of data obtained in MAGL KO and WT mice demonstrated a significant interaction between treatment and genotype in nicotine withdrawal [F(1, 20) = 126.7; P < 0.001]. Specifically, mecamylamine did not precipitate nicotine somatic withdrawal signs in MAGL KO mice (Figure 3). No difference between male and female mice in response to nicotine withdrawal was found (data not shown). Furthermore, MAGL WT and KO mice did not show significant differences in acute responses to nicotine, such as antinociception [vehicle WT mice = 11 ± 5% analgesia; nicotine (2.5 mg·kg^{-1}) WT mice = 78 ± 16% analgesia; vehicle KO mice = 12 ± 6% analgesia; nicotine (2.5 mg·kg^{-1}) WT mice = 76 ± 8% analgesia] and hypothermia [vehicle WT mice = 0.2 ± 0.1°C; nicotine (2.5 mg·kg^{-1}) WT mice = –3.4 ± 0.3°C; vehicle KO mice = 0.4 ± 0.3°C; nicotine (2.5 mg·kg^{-1}) WT mice = –3.5 ± 0.2°C]. These results suggest that the absence of nicotine somatic signs of withdrawal in the MAGL KO mice is not due to a lack of pharmacological sensitivity to nicotine.

The CB_1 receptor antagonist rimonabant was administered to assess whether the attenuation of somatic withdrawal signs in nicotine-dependent mice by JZL184 (8 mg·kg^{-1}, i.p.) blockade is mediated by CB_1 receptors. Mice infused with nicotine (24 mg·kg^{-1}·day^{-1}) or vehicle for 14 days were challenged with mecamylamine following pretreatment with rimonabant (0.3 and 3 mg·kg^{-1}, i.p.) and JZL184 (8 mg·kg^{-1}, i.p) on day 15. As shown in Figure 4, rimonabant prevented JZL184-mediated attenuation of mecamylamine-precipitated withdrawal signs [F(10, 68) = 9.1, P < 0.0001].

Inhibition of MAGL attenuates spontaneous nicotine somatic withdrawal signs

Mice were made dependent upon nicotine (24 mg·kg^{-1}·day^{-1}) via minipump infusion for 14 days, and on the evening of day 14, the minipumps were removed. As expected, nicotine-dependent mice showed a significant increase in somatic withdrawal signs compared with the saline-treated group (Table 2). Similar to the results with precipitated withdrawal, pretreatment with JZL184, 2 h prior to observation, produced

Table 1

JZL184 increased 2-AG levels in specific brain regions

A					
Treatment	**2-AG levels (nmol·g^{-1})**				
	PFC	**HIP**	**AMG**	**VTA**	**NAc**
Vehicle	5.3 ± 1.0	9.0 ± 1.0	16.5 ± 3.0	4.4 ± 1.0	3.6 ± 1.3
JZL184	37.7 ± 6.0*	31.6 ± 7.0*	64.6 ± 5.0*	29.3 ± 7.0*	34.9 ± 6.0*
B					
Treatment	**AEA levels (pmol·g^{-1})**				
	PFC	**HIP**	**AMG**	**VTA**	**NAc**
Vehicle	21.6 ± 4.2	29.5 ± 1.9	36.1 ± 5.7	6.63 ± 1.2	51.6 ± 11.2
JZL184 (8)	15.9 ± 0.7	36.2 ± 10.5	31.6 ± 4.2	14.2 ± 4.6	58.2 ± 5.8
C					
Treatment	**AA levels (nmol·g^{-1})**				
	PFC	**Hip**	**AMG**	**VTA**	**NAc**
Vehicle	167.0 ± 29.3	154.7 ± 18.7	177.1 ± 24.7	37.8 ± 5.3	152.2 ± 20.8
JZL184 (8)	136.5 ± 13.9	147.7 ± 23.5	145.4 ± 13.4	40.5 ± 7.8	156.9 ± 19.5

Mice were administered either vehicle or JZL184 (8 mg·kg^{-1}, i.p.) 2 h before brain regions were collected and levels of 2-AG levels (A), AEA (B) and AA (C) were measured. Data are expressed as mean ± SEM. *P < 0.05 versus vehicle. n = 4–6 mice per group. 2-AG, 2-arachidonylglycerol; AA, arachidonic acid; AEA, arachidonoylethanolamine; AMG, amygdala; Hip, hippocampus; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

significant and dose-related attenuation of nicotine somatic withdrawal signs [F(5, 33) = 22.3; P < 0.0001].

Repeated administration of JZL184 did not produce tolerance to its effect on nicotine withdrawal

The actions of JZL184 (8 mg·kg^{-1}, i.p.) to alleviate nicotine-precipitated withdrawal effects did not undergo tolerance following 6 days of repeated treatment [F(11, 72) = 22.3; P < 0.0001]. Similar to the data observed in Figure 2, JZL184 blocked the expression of somatic withdrawal signs in nicotine-dependent mice regardless of whether it was the first treatment or after 6 days of repeated administration (Figure 5). In the absence of mecamylamine, none of the groups displayed significant increases in withdrawal signs. Furthermore, repeated JZL184 (8 mg·kg^{-1}, i.p.) did not induce a withdrawal effect in saline-infused mice treated with mecamylamine (2 mg·kg^{-1}, s.c.). Moreover, as shown in Table 3, CB_1 receptor levels were not significantly affected by repeated administration of this behaviourally active dose of JZL184 compared with vehicle control groups in the AMG (P = 0.2), NAc (P = 0.8) or VTA (P = 0.2). Furthermore, CB_1 receptor function, as assessed in the CP55,940-stimulated [^{35}S]GTPγS binding assay, was not altered by repeated treatment with JZL184 (8 mg·kg^{-1}, i.p.), compared with vehicle in the AMG (P = 0.2), NAc (P = 0.7) or VTA (P = 0.5) (Table 4).

MAGL inhibition reduces CPA in nicotine-dependent mice undergoing withdrawal

As nicotine withdrawal also elicits affective signs, we evaluated whether MAGL inhibition would prevent the development of CPA associated with nicotine withdrawal. A two-way ANOVA (JZL184 × Mecamylamine) revealed a significant interaction between JZL184 and mecamylamine [F(1, 34) = 5.5; P < 0.02], indicating that JZL184 (8 mg·kg^{-1}, i.p.) prevented the development of mecamylamine-induced CPA (Figure 6A). Neither JZL184 nor mecamylamine significantly affected responses in saline-infused mice (P = 0.1) (Figure 6A).

In the final experiment, we assessed whether prevention of mecamylamine-induced CPA by JZL184 was CB_1 receptor-mediated. Rimonabant (3 mg·kg^{-1}, i.p.) prevented the effect of JZL184 (8 mg·kg^{-1}, i.p.) on mecamylamine-induced CPA [F(6, 57) = 7.0; P < 0.0001] (Figure 6B). The dose of rimonabant used in this assessment did not significantly affect responses in saline-infused mice in the CPA test and did not alter nicotine withdrawal aversion in nicotine-dependent mice (Figure 6B).

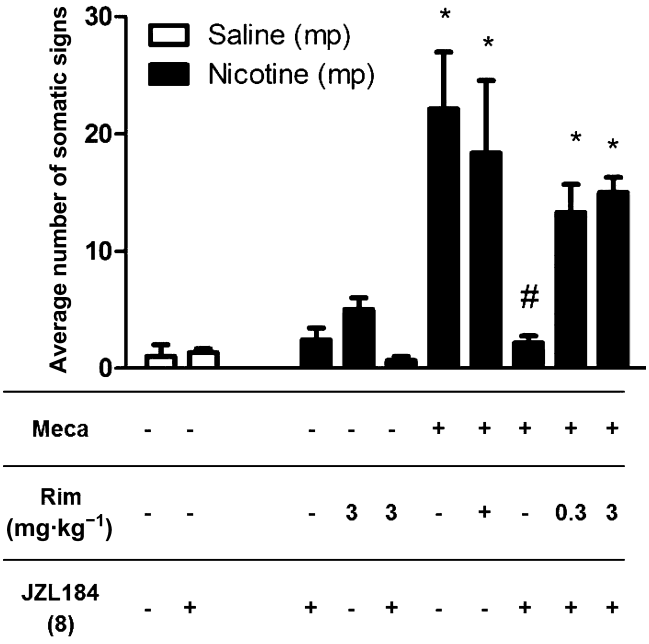

Figure 4

JZL184-mediated reduction in nicotine somatic signs is CB_1 receptor-mediated. Rimonabant (0.3 and 3 mg·kg^{-1}, i.p.) administered 90 min after JZL184 (8 mg·kg^{-1}, i.p.) blocked the effects of JZL184 on nicotine somatic signs. Data are expressed as mean ± SEM of n = 6–8 mice per group. *P < 0.05 versus all control groups.

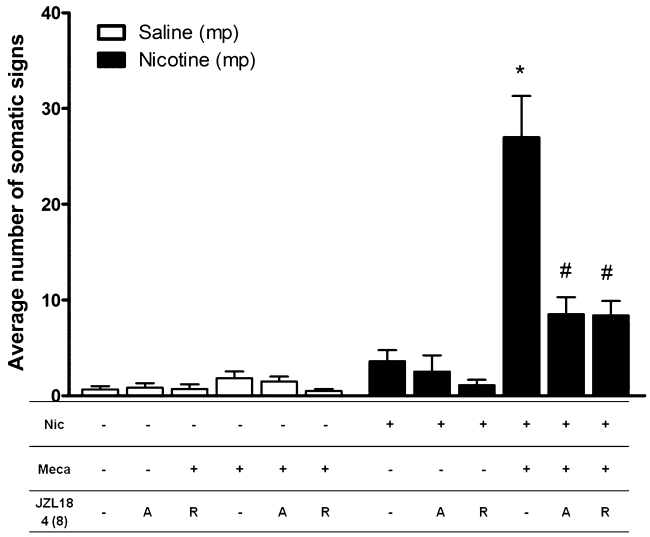

Figure 5

Repeated administration of JZL184 decreases nicotine somatic withdrawal signs. Mice implanted with 14 day minipumps were chronically infused with either nicotine or saline. On day 9 of minipump infusion, the mice were divided into two groups. The first group received JZL184 (8 mg·kg^{-1}, i.p.) for 6 days, whereas the second group received vehicle (i.p.) for 5 days and on day 15 was administered JZL184 (8 mg·kg^{-1}, i.p.) or vehicle. The results show that repeated JZL184 administration decreased the number of somatic signs during nicotine withdrawal. A = acute; R = repeated. Data are expressed as mean ± SEM of n = 6–8 mice per group. *P < 0.05 versus all control groups; #P < 0.05 versus nicotine (mp) + mecamylamine (2 mg·kg^{-1}). mp = minipump.

Table 2

Acute MAGL inhibition by JZL184 attenuated spontaneous nicotine somatic withdrawal signs

Groups	Average number (±SEM) of somatic signs
Saline (mp)/Vehicle	2.2 ± 1.0
Saline (mp)/JZL184 (8)	1.3 ± 0.3
Nicotine (mp)/Vehicle	18.8 ± 1.9*
Nicotine (mp)/JZL184 (2)	15.3 ± 0.8*
Nicotine (mp)/JZL184 (4)	12.7 ± 1.9*
Nicotine (mp)/JZL184 (8)	8.8 ± 1.3#

Mice received nicotine or saline via osmotic minipumps (24 mg·kg^{-1}·day^{-1}) for 14 days. Minipumps were removed on the evening of day 14. On day 15, 18–24 h after minipump removal, mice were given an i.p. injection of either vehicle or JZL184 (2, 4, or 8 mg·kg^{-1}) and 2 h later were observed for somatic withdrawal signs for 30 min. The total withdrawal signs measure consists of paw tremors, head shakes, backing, ptosis and jumping. Data are expressed as mean ± SEM of n = 6 mice per group. *P < 0.05 versus control groups; #P < versus Nicotine (mp)/Vehicle; Nicotine (mp)/JZL184 (2); and Nicotine (mp)/JZL184 (4).

Polymorphisms in the human MAGL *gene are associated with nicotine withdrawal*

Association analyses conducted on four separate data sets consisting of over 7000 subjects from African-American and European-American ethnic groups revealed significant correlations between FTND2 (i.e. which cigarette you hate most to give up?) and several SNPs (Table 5). Of these markers, rs549662 remained significant after multiple testing correction. Although it did not achieve significance with FTND1 (How soon after you wake up do you smoke your first cigarette?) or FTND3 (Do you find it difficult to refrain from smoking in places where it is forbidden), the allele had the same effect (i.e. negative correlation) for both phenotypes. The significant marker rs549662 is located in the intron of the *MAGL* gene between the second and third exon. ENCODE data from UCSC genome browser (http://www.genome.ucsc.edu/) indicated that this marker is located in a region with high H3K27Ac modification; therefore, it might be involved in the regulation of MAGL expression.

Discussion

In the present study, genetic correlation analyses of nicotine withdrawal phenotype data and gene expression data across

Table 3

Repeated treatment with JZL184 (8 mg/kg, i.p.) does not lead to CB_1 receptor down-regulation in NAc, AMG or VTA during nicotine-precipitated withdrawal

Group	Saline (mp) + Vehicle	Saline (mp) + JZL184(8)	Nicotine (mp) + JZL184 (8)	Nicotine (mp) + 1:1:18 + Meca(2)	Nicotine (mp) + JZL184 (8) + Meca (2)
AMG					
B_{max} (pM)	1.6 ± 0.3	1.2 ± 0.4	1.2 ± 0.3	2.8 ± 0.6	1.5 ± 1.0
K_d (nM)	1.1 ± 0.3	0.7 ± 0.1	1.1 ± 0.4	2.0 ± 0.4	1.1 ± 0.8
NAc					
B_{max} (pM)	0.9 ± 0.2	1.1 ± 0.3	1.4 ± 0.8	1.0 ± 0.1	0.9 ± 0.3
K_d (nM)	1.8 ± 0.7	1.4 ± 0.5	5.2 ± 4.5	0.8 ± 0.2	2.4 ± 1.1
VTA					
B_{max} (pM)	1.7 ± 0.5	0.9 ± 0.2	1.5 ± 0.4	1.62 ± 0.7	0.6 ± 0.1
K_d (nM)	5.3 ± 2.3	1.5 ± 0.4	5.7 ± 2.1	9.1 ± 8.5	1.0 ± 0.3

Mice were implanted with nicotine (24 mg·kg^{-1}) or saline osmotic minipumps (mp) for 9 days and were then treated with JZL184 (8 mg·kg^{-1}, i.p.) or vehicle (i.p.) for 6 days. On day 15, mice were given an s.c. injection of either vehicle or mecamylamine (Meca; 2 mg·kg^{-1}) and 30 min later brain regions were dissected. Data are reported as mean ± SEM of four separate experiments per group that were each performed in duplicate. AMG, amygdala; NAc, nucleus accumbens; VTA, ventral tegmental area.

Table 4

Repeated treatment with JZL184 does not produce CB_1 receptor desensitization in NAc, AMG or VTA during nicotine-precipitated withdrawal

Group	Saline (mp) + Vehicle	Saline (mp) + JZL184	Nicotine (mp) + JZL184	Nicotine (mp) + 1:1:18 + Meca (2)	Nicotine (mp) + JZL184 + Meca (2)
AMG					
E_{max} (% stimulation)	70.0 ± 3.1	50.0 ± 7.5	47.4 ± 2.5	63.4 ± 11.7	46.4 ± 4.1
EC_{50} (nM)	22.0 ± 7.7	34.3 ± 3.4	33.8 ± 6.0	11.8 ± 4.0	25.5 ± 11.3
NAc					
E_{max} (% stimulation)	52.2 ± 8.4	52.4 ± 4.0	35.8 ± 5.0	46.93 ± 8.7	42.4 ± 2.8
EC_{50} (nM)	40.9 ± 8.7	34.1 ± 13.0	23.4 ± 4.8	22.8 ± 5.1	39.7 ± 23.5
VTA					
E_{max} (% stimulation)	35.0 ± 4.8	36.7 ± 7.1	28.1 ± 4.6	39.1 ± 11.0	29.7 ± 1.9
EC_{50} (nM)	53.9 ± 21.9	46.7 ± 32.2	49.9 ± 14.7	21.7 ± 8.8	39.6 ± 19.7

Mice were implanted with nicotine (24 mg·kg^{-1}) or saline osmotic minipumps (mp) for 9 days and were then treated with JZL184 (8 mg·kg^{-1}, i.p.) or vehicle (i.p.) for 6 days. On day 15, mice were given an s.c. injection of either vehicle or mecamylamine (Meca; 2 mg·kg^{-1}), and 30 min later, brain regions were dissected. Data are reported as mean ± SEM of four separate experiments per group that were each performed in triplicate. AMG, amygdala; NAc, nucleus accumbens; VTA, ventral tegmental area.

the BXD panel suggested a modulatory role of MAGL in nicotine withdrawal. Strikingly, pharmacological inhibition of enzyme activity or genetic deletion of MAGL reduced precipitated and spontaneous somatic as well as affective withdrawal signs in nicotine-dependent mice via a CB_1 receptor-mediated mechanism. Furthermore, tolerance did not develop to JZL184-mediated alleviation of nicotine somatic withdrawal signs after repeated administration of JZL184 (8 mg·kg^{-1}) and CB_1 receptors and receptor-mediated G-protein activity were not altered in the NAc, AMG or VTA by repeated JZL184 administration. Additionally, a variant of the human *MAGL* gene associated with nicotine withdrawal raises the provocative possibility that this enzyme contributes to nicotine addiction in humans.

Basal MAGL mRNA levels significantly positively correlate with mecamylamine-induced somatic signs in both whole brain and fore/midbrain data sets, suggesting that increased levels of MAGL, and concomitant reductions in 2-AG, may contribute to the magnitude of nicotine physical withdrawal signs. Thus, decreased MAGL expression might be protective against nicotine withdrawal signs by enhancing the activity of the endocannabinoid system. The finding that THC attenuated nicotine withdrawal signs in mice through a CB_1 receptor mechanism of action (Balerio *et al.*, 2004) further

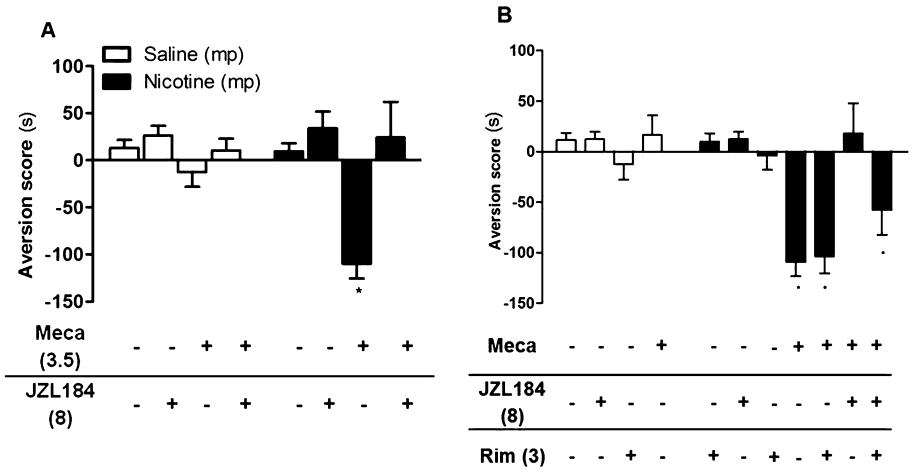

Figure 6

JZL184 prevents conditioned place aversion (CPA) caused by nicotine-precipitated withdrawal through a CB_1 receptor mechanism of action. (A) Mecamylamine (Meca; 3.5 mg·kg^{-1}, s.c.) precipitated significant CPA in mice infused chronically with nicotine (Nic; 36 mg·kg^{-1}) via osmotic minipumps. Development of aversion was blocked by pretreatment of JZL184 (8 mg·kg^{-1}, i.p.). (B) Blockade of CPA by JZL184 (8 mg·kg^{-1}, i.p.) was prevented by pretreatment with rimonabant (Rim; 3 mg·kg^{-1}, i.p.) administered 90 min after JZL184. Data are expressed as mean ± SEM of n = 8–10 mice per group. *P < 0.05 versus all treatment groups.

Table 5

MAGL SNP association with nicotine withdrawal

	SNP	Reference allele	Reference allele frequency	OR/BETA	SE	OR_95L	OR_95U	Z	P-value
FTND1	rs567384	G	0.117	–0.03	0.0533	–0.14	0.07	–0.61	0.5433
	rs608318	C	0.185	–0.02	0.0250	–0.07	0.03	–0.69	0.4887
	rs7635934	G	0.178	–0.03	0.0245	–0.08	0.02	–1.30	0.1938
	rs549662	C	0.191	–0.05	0.0408	–0.13	0.03	–1.34	0.1804
	rs604300	T	0.083	0.14	0.0591	0.02	0.25	2.30	0.0213
FTND2	rs567384	G	0.117	0.87	0.0495	0.77	0.98	–2.37	0.0180
	rs608318	C	0.185	0.86	0.0639	0.74	1.01	–1.84	0.0655
	rs7635934	G	0.178	0.91	0.0440	0.83	1.01	–1.81	0.0701
	rs549662	**C**	**0.191**	**0.76**	**0.0534**	**0.65**	**0.88**	**–3.68**	**0.0002**
	rs604300	T	0.083	1.35	0.1287	1.10	1.66	2.83	0.0046
FTND3	rs567384	G	0.117	0.97	0.1024	0.77	1.23	–0.22	0.8290
	rs608318	C	0.185	1.06	0.0853	0.89	1.25	0.61	0.5395
	rs7635934	G	0.178	0.98	0.0712	0.84	1.15	–0.21	0.8323
	rs549662	C	0.191	0.88	0.0659	0.75	1.03	–1.61	0.1073
	rs604300	T	0.083	1.27	0.1942	0.89	1.81	1.32	0.1862

Association analyses were conducted on four separate data sets consisting of over 7000 subjects from African-American and European-American ethnic groups. Phenotypes measured were FTND1 (How soon after you wake up do you smoke your first cigarette?), FTND2 (i.e., which cigarette you hate most to give up?) and FTND3 (Do you find it difficult to refrain from smoking in places where it is forbidden). FTND, Fagerstrom Test for Nicotine Dependence; L, lower limit; OR/BETA, odd ratio/beta (standardized regression coefficients); SNP, single-nucleotide polymorphism; U, upper limit; *Z*, *Z*-value. Bold values are found to be significant after multiple testing corrections.

supports this possibility. Here, we report that acute administration of JZL184 dose-dependently blocked precipitated nicotine somatic withdrawal signs, which included paw tremors, head shakes, body tremors and retropulsion. In agreement, MAGL KO mice implanted with nicotine minipumps did not exhibit somatic withdrawal signs even after mecamylamine challenge. Likewise, JZL184 blocked spontaneous somatic withdrawal signs in mice elicited by removal

of nicotine Alzet minipumps on day 14. Rimonabant prevented the protective effects of JZL184 (8 mg·kg^{-1}, i.p.) on mecamylamine-precipitated somatic withdrawal signs, suggesting a CB_1 receptor-mediated mechanism. Similarly, JZL184 significantly decreased mecamylamine-induced aversion in the CPA test in a CB_1 receptor-dependent manner. These results support the hypothesis that increasing brain 2-AG levels by MAGL inhibition blocks the expression of somatic and affective signs of nicotine withdrawal through activation of CB_1 receptors. While these results do not exclude a possible role for CB_2 receptors because 2-AG activates both CB receptor subtypes, our recently published paper demonstrating that CB_2 receptors are not necessary for nicotine withdrawal (Ignatowska-Jankowska *et al.*, 2013) does not strongly suggest this possibility.

The results with the MAGL KO mice are generally consistent with JZL184 blockade of nicotine withdrawal. These animals were reported earlier to show the expected increase in brain 2-AG levels, decrease in brain AA levels and no change in anandamide levels (Schlosburg *et al.*, 2010). However, a heterogeneous reduction in CB_1 receptor function throughout the brain was seen in the MAGL KO mouse, which is consistent with chronic but not acute (similar to our testing with acute JZL184 in nicotine-dependent mice) MAGL blockade by JZL184.

Analysis of the BXD recombinant mouse line yielded a significant positive correlation between MAGL expression and the magnitude of physical withdrawal signs in nicotine-dependent mice. These data are consistent with the idea that increased 2-AG levels may alleviate nicotine withdrawal signs. In support of this idea, JZL184 (8 mg·kg^{-1}), which blocked both nicotine withdrawal somatic and affective signs, selectively increased 2-AG levels in the PFC, NAc, HIP, AMG, and VTA, without affecting AEA or AA levels in these brain regions. Interestingly, 2-AG level increase produced by JZL184 was not uniform among brain regions. JZL184 increased 2-AG levels by the greatest magnitude (10-fold) in the NAc, while the lowest fold increase in 2-AG levels was found in the AMG and Hip (~4-fold). The observation that JZL184 increased brain 2-AG levels, but not AEA levels, is consistent with its preferential blockade of MAGL over FAAH. It is of consequence that JZL-184 was 500-fold more potent as a MAGL inhibitor (IC_{50} = 8 nM) than as a FAAH inhibitor (IC_{50} = 4000 nM) (Long *et al.*, 2009a), and its acute administration does not affect AEA levels (Long *et al.*, 2009a; Schlosburg *et al.*, 2010). The lack of altered AA levels in each brain region examined is relevant because MAGL is known to play an important regulatory role in the biosynthesis of AA in brain, which serves as a precursor of other lipid signalling molecules through COX and other enzymes (Long *et al.*, 2009a; Schlosburg *et al.*, 2010; Nomura *et al.*, 2011). Thus, the brain lipid data are consistent with the idea that the alleviation of withdrawal effects by JZL184 in nicotine-dependent mice was mediated by elevated levels of 2-AG. Accordingly, 2-AG may activate CB_1 receptors present presynaptically on GABAergic interneurons in the VTA, thereby disinhibiting dopamine neurons and resulting in a net increase of dopamine release in the NAc (Maldonado *et al.*, 2006). This mechanism could possibly explain the reduction of nicotine somatic and aversive withdrawal signs by JZL184. Indeed, dopaminergic neuronal activity in the VTA (Liu and Jin, 2004) and decreased dopamine output in the NAc are believed to contribute to nicotine withdrawal (Hildebrand *et al.*, 1998; Rada *et al.*, 2001; Zhang *et al.*, 2012).

As repeated administration of high (>16 mg·kg^{-1}), but not low (<8 mg·kg^{-1}), doses of JZL184 can result in tolerance and concomitant reductions in expression of CB_1 receptors in brain (Schlosburg *et al.*, 2010; Kinsey *et al.*, 2013), we evaluated whether repeated administration of 8 mg·kg^{-1} JZL184 would retain its actions in reducing nicotine withdrawal. Interestingly, tolerance did not develop to JZL184-mediated alleviation of nicotine somatic withdrawal signs following repeated JZL184 (8 mg·kg^{-1}, i.p., over 6 days) administration in mice. Consistent with this observation, CB_1 receptor desensitization and down-regulation, which are thought to contribute to tolerance (Nguyen *et al.*, 2012), were not found in the brain regions examined in JZL184-treated mice. Our results are in agreement with recent reports demonstrating that the anxiolytic-like and antinociceptive effects of lower doses of JZL184 (i.e. 8 mg·kg^{-1}) are maintained after 6 days of daily administration in both rats and mice (Sciolino *et al.*, 2011; Ghosh *et al.*, 2013; Kinsey *et al.*, 2013).

Interestingly, the effect of MAGL inhibition on nicotine withdrawal reported in the present study is in marked contrast to the augmented severity of nicotine withdrawal responses displayed by FAAH-mutant mice (Merritt *et al.*, 2008). It is plausible that elevated 2-AG levels activate different CB_1 receptor containing neuronal circuits from those activated by Alternatively, AEA might augment nicotine withdrawal signs through actions at TRPV1 receptors or other substrates of FAAH, such as PEA and OEA, might be driving these actions (Ahern, 2003; Fu *et al.*, 2003; Muldoon *et al.*, 2013). Importantly, URB597, a relatively selective FAAH inhibitor, decreased anxiety-like behaviours but not somatic signs in rats undergoing nicotine withdrawal (Cippitelli *et al.*, 2011). However, it is still unknown if species differences exist with MAGL inhibition in nicotine withdrawal.

The blockade of nicotine withdrawal responses in JZL184-treated WT mice or MAGL KO mice suggests that blockade of this enzyme may provide a novel strategy to treat nicotine withdrawal. The significant association of the *MAGL* gene with smoking withdrawal in humans further supports this idea. Interestingly, the ENCODE data suggest that rs549662 is located in a region enriched in histone acetylation (H3K27Ac), indicating that this region is likely to be involved in the transcription regulation of *MAGL* gene (Dunham *et al.*, 2012).

Overall, the results of the present study suggest that MAGL inhibitors could have therapeutic utility in reducing both somatic and affective signs of nicotine withdrawal. JZL184 has also been shown to block physical signs of THC and morphine withdrawal via a CB_1 receptor-dependent mechanism (Schlosburg *et al.*, 2009; Ramesh *et al.*, 2011). The current results support the idea that MAGL plays a role in drug dependence, possibly through enhancing 2-AG levels in specific regions of the mesolimbic/limbic systems, which results in enhanced activation of CB_1 receptors in these regions. Collectively, these findings suggest that inhibition of MAGL represents a promising new target for the development of pharmacotherapies to treat dependence from multiple classes of drugs.

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Authorship contributions

P. P. M., J. L. H., J. C., X. C., A. H. L. and M. I. D. participated in research design. P. P. M., J. C., J. L. H. and R. A. A. conducted experiments. B. F. C. contributed new reagents or analytical tools. P. P. M., J. C., J. L. H., R. A. A., X. C., A. H. and M. I. D performed data analysis. P. P. M., J. C., J. L. H., R. A. A., L. J. S-S., B. F. C., M. F. M., X. C., A. H. L. and M. I. D. wrote or contributed to the writing of the manuscript.

Conflict of interest

All of the authors, except Dr Aron Lichtman and Dr Benjamin Cravatt, declare no conflict of interest.

Dr Aron Lichtman has received compensation as consultant from Iron Pharmaceuticals and Abide Therapeutics for work unrelated to this publication.

Dr Cravatt is a scientific advisor to Abide Therapeutics, a company interested in developing serine hydrolase inhibitors as therapeutic agents.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Table S1 Nicotine or saline-infused mice do not display somatic signs after JZL184 administration. Mice received nicotine or saline via osmotic minipumps (24 mg·kg^{-1}·day^{-1}) for 14 days. On day 15, mice were given an i.p. injection of either vehicle or JZL184 (4, 8 and 40 mg·kg^{-1}) and 2 h later were observed for somatic withdrawal signs for 20 min. The total withdrawal signs measure consists of paw tremors, head shakes, backing, ptosis and jumping. Data are expressed as mean ± SEM of n = 6 mice per group. mp = minipump.