

The Novel Metabotropic Glutamate Receptor 2 Positive Allosteric Modulator, AZD8529, Decreases Nicotine Self-Administration and Relapse in Squirrel Monkeys

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ABSTRACT

BACKGROUND: Based on rodent studies, group II metabotropic glutamate receptors (mGluR2 and mGluR3) were suggested as targets for addiction treatment. However, LY379268 and other group II agonists do not discriminate between the mainly presynaptic inhibitory mGluR2 (the proposed treatment target) and mGluR3. These agonists also produce tolerance over repeated administration and are no longer considered for addiction treatment. Here, we determined the effects of AZD8529, a selective positive allosteric modulator of mGluR2, on abuse-related effects of nicotine in squirrel monkeys and rats.

METHODS: We first assessed modulation of mGluR2 function by AZD8529 using functional in vitro assays in membranes prepared from a cell line expressing human mGluR2 and in primate brain slices. We then determined AZD8529 (.03–10 mg/kg, intramuscular injection) effects on intravenous nicotine self-administration and reinstatement of nicotine seeking induced by nicotine priming or nicotine-associated cues. We also determined AZD8529 effects on food self-administration in monkeys and nicotine-induced dopamine release in accumbens shell in rats.

RESULTS: AZD8529 potentiated agonist-induced activation of mGluR2 in the membrane-binding assay and in primate cortex, hippocampus, and striatum. In monkeys, AZD8529 decreased nicotine self-administration at doses (.3–3 mg/kg) that did not affect food self-administration. AZD8529 also reduced nicotine priming- and cue-induced reinstatement of nicotine seeking after extinction of the drug-reinforced responding. In rats, AZD8529 decreased nicotine-induced accumbens dopamine release.

CONCLUSIONS: These results provide evidence for efficacy of positive allosteric modulators of mGluR2 in nonhuman primate models of nicotine reinforcement and relapse. This drug class should be considered for nicotine addiction treatment.

Keywords: Allosteric modulation, Glutamate, Nicotine, Relapse, Self-administration, Smoking cessation

<http://dx.doi.org/10.1016/j.biopsych.2015.01.014>

Tobacco smoking, the leading cause of preventable death, is primarily driven by nicotine (1,2). Several medications currently are available for smoking cessation (varenicline, bupropion, nicotine replacement), but high relapse rates are observed during and after treatment (3–5). Novel treatments to prevent relapse are needed. In rodents, manipulations of metabotropic glutamate receptors (mGluRs) decrease nicotine-induced potentiation of brain stimulation reward and nicotine withdrawal symptoms (6). Based on these and related findings, group II mGluRs have been considered potential targets for nicotine addiction treatment (7). Group II mGluRs consist of mGluR2 and mGluR3 subtypes (8). The mGluR2s are expressed primarily on presynaptic glutamate neurons, and their activation leads to decreased evoked glutamate release (8,9). The mGluR3s are expressed on postsynaptic and presynaptic neurons and on glia (8,10). The prototype drug

used to assess the function of group II mGluRs is LY379268, an orthosteric agonist that binds to both mGluR2s and mGluR3s (8,11).

In rats, systemic injections of LY379268 or related mGluR2/mGluR3 agonists decrease discriminative cue-induced reinstatement of cocaine seeking and context-induced reinstatement of heroin seeking (12,13), discrete cue-induced reinstatement of heroin seeking (14), cocaine priming-induced reinstatement (15), spontaneous recovery of alcohol seeking (16), cue-induced cocaine seeking (17), and discrete cue-induced and drug priming-induced reinstatement of methamphetamine seeking (18). LY379268 injections also decrease cocaine priming-induced reinstatement in squirrel monkeys (19). Finally, LY379268 injections decrease nicotine self-administration and discrete cue-induced reinstatement of nicotine seeking (20).

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However, from a medication development perspective, there are limitations with LY379268 and related orthosteric agonists. LY379268 has low bioavailability (21), and tolerance develops to its effects (20). Additionally, LY379268 activates the mGluR3 subtype whose physiologic functions are unknown (8). These limitations led to development of selective positive allosteric modulators (PAMs) of mGluR2 (22–24). The PAMs of mGluR2 bind to an allosteric site of the receptor and facilitate agonist-mediated receptor activity (25,26).

Jin *et al.* (27) reported that a selective PAM of mGluR2, BINA, decreases cocaine self-administration and cue-induced reinstatement. They also reported that a BINA analogue with superior pharmacokinetic properties and brain penetration decreases nicotine self-administration in rats (21). Based on these studies, we used our squirrel monkey model (28,29) to determine the effects of AZD8529, a selective PAM for mGluR2 (30), on nicotine self-administration and relapse to nicotine seeking, as assessed in the reinstatement procedure (31). We also provide results from *in vivo* and *in vitro* assays on the selectivity of AZD8529 to mGluR2 and results on the drug's effect on nicotine-induced dopamine release in nucleus accumbens shell.

METHODS AND MATERIALS

Subjects

For the autoradiography experiment, we used three male 5- to 6-year-old cynomolgus monkeys (*Macaca fascicularis*; Covance, Inc, Denver, Pennsylvania). The AstraZeneca (Wilmington, Delaware) animal care and use committee approved the experiment, and procedures were performed in accordance with the AstraZeneca Global Research and Development animal care standards.

For the behavioral experiment, we used 9- to 13-year-old male squirrel monkeys (*Saimiri sciurea*), weighing 750–1050 g. The monkeys had been trained to self-administer nicotine or food before the study and had no self-administration history with other drugs. We implanted intravenous catheters as previously described (32). The monkeys wore nylon-mesh jackets to protect these catheters. Each weekday, we flushed the catheters, refilled them with saline, and sealed them with obturators. For microdialysis, we used male Sprague Dawley rats (300–350 g; Charles River Laboratories, Inc, Wilmington, Massachusetts). Squirrel monkeys were housed individually, and rats were group-housed at the National Institute on Drug Abuse Intramural Research Program facility (regular 12-hour light/dark cycle). The National Institute on Drug Abuse Intramural Research Program animal care and use committee approved the experiments, which were carried out in accordance with the 2003 National Research Council Guidelines.

Functional mGluR2 Assays

Receptor Selectivity Assay. To determine the selectivity of AZD8529 within the mGluR family, we used fluorescence-based assays (33,34) and HEK 293 cell lines expressing human mGluR constructs. The cell lines expressed chimeric fusion constructs hmGluR2/hCaR*, hmGluR1/hCaR*, hmGluR3/hCaR*, hmGluR4/hCaR*, hmGluR5/hCaR*, hmGluR6/

hCaR*, hmGluR7/hCaR*, and hmGluR8/hCaR*, each including the extracellular domain and transmembrane domain of human mGluR and the intracellular domain of the human calcium receptor fused to the promiscuous chimeric protein Gqi5 as described previously (35).

Receptor Screening. We evaluated AZD8529 at 10 $\mu\text{mol/L}$ for off-target effects using radioligand binding assays (MDS Pharma Services, Bothell, Washington) based on published methods. We ran reference standards for each assay. We determined inhibitory concentration of 50% (IC_{50}) values using nonlinear, least squares regression analysis of the Data Analysis Toolbox (MDL Information Systems, formerly of San Leandro, California).

[³⁵S]GTP γ S Binding Human mGlu2-CHO Membranes.

We used membranes prepared from a Chinese hamster ovary (CHO) cell line expressing the human mGluR2 and performed the assay in a scintillation proximity assay format. We grew CHO cells expressing the human mGluR2 to ~80% confluence, washed the cells in ice-cold phosphate-buffered saline, and stored them frozen until membrane preparation. Assay buffer contained .05 mol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, .10 mol/L sodium chloride, and .01 mol/L magnesium chloride, pH 7.4 plus 100 $\mu\text{mol/L}$ dithiothreitol and 3 $\mu\text{mol/L}$ guanosine diphosphate (GDP). We started the assay by adding a mixture of wheat germ agglutinin scintillation proximity assay beads (.75 mg/mL) and membranes (6 $\mu\text{g/mL}$) in assay buffer containing AZD8529 or vehicle. After 15-min incubation, we added a solution containing the [³⁵S]GTP γ S and L-glutamate (final concentrations 100 pmol/L [³⁵S]GTP γ S and 0–100 $\mu\text{mol/L}$ glutamate). After incubation at room temperature (60 min), we centrifuged the assay plates and read them on the TopCount scintillation counter (Perkin Elmer, Waltham, Massachusetts). We determined [³⁵S]GTP γ S binding in the absence of glutamate and in the presence of 100- $\mu\text{mol/L}$ glutamate as 0% and 100% levels, respectively. We estimated the modulator activity of AZD8529 on mGluR2 activation from the concentration response curves of AZD8529 fitted with a four-parameter logistic equation to calculate the apparent potency (EC_{50}) and maximal efficacy (E_{max}).

[³⁵S]GTP γ S Autoradiography in Cynomolgus Monkey Brain Slices.

We anesthetized the monkey with sodium pentobarbital (100 mg/kg), perfused it with saline, and then removed the brain and froze it in cooled isopentane. We cut 20- μm striatum and hippocampus sections on a cryostat, mounted the sections on glass slides, and stored them at -80°C until use. We warmed the sections to room temperature in a vacuum chamber over 3 hours on the day of the experiment. We incubated the sections in 50 mmol/L Tris HCl, 3 mmol/L magnesium chloride, .2 mmol/L ethylene glycol tetraacetic acid, 100 mmol/L sodium chloride, and 0.2 mmol/L dithiothreitol (Tris assay buffer [TAB]), pH 7.4 at 25°C for 10 min. We then incubated the slides in TAB containing 2 mmol/L GDP for 15 min at 25°C . We placed the slides in one of the following four conditions for 2 hours at 25°C : basal, TAB + 2 mmol/L GDP + .04 nmol/L [³⁵S]GTP γ S; agonist alone, TAB + 2 mmol/L GDP + .04 nmol/L [³⁵S]

GTP γ S + 1 μ mol/L LY379268; modulator alone, TAB + 2 mmol/L GDP + .04 nmol/L [35 S]GTP γ S + 3 μ mol/L AZD8529; modulator + agonist, TAB + 2 mmol/L GDP + .04 nmol/L [35 S]GTP γ S + 1 μ mol/L LY379268 + 3 μ mol/L AZD8529; modulator + agonist + antagonist, TAB + 2 mmol/L GDP + .04 nmol/L [35 S]GTP γ S + 1 μ mol/L LY379268 + 3 μ mol/L AZD8529 + 1 μ mol/L LY341495. We washed the sections two times in 4°C 50 mmol/L Tris HCl, pH 7.4, 5 min each, and rinsed them in ice-cold water and air dried the slides. We exposed the slides to BioMax MR film (Kodak, Rochester, New York) for 2 days and developed (using standard techniques), digitized, and analyzed.

Behavioral Studies in Squirrel Monkeys

Apparatus. We performed the experiments in sound-attenuating isolation chambers each equipped with a Plexiglas chair, a house light, and white noise for masking of external sound. The chair contained a response lever (BRS/LVE Corp., Laurel, Maryland) mounted on a transparent front wall; each press of the lever with a force $>.2$ N produced an audible click and was recorded as a response. Pairs of green and amber stimulus lights, mounted behind the transparent front wall of the chair, could be illuminated and used as visual cues. We connected the monkey's catheter to polyethylene tubing, which passed out of the isolation chamber where we attached it to a motor-driven syringe pump. The self-administration chambers were controlled through a Med Associates interface and MED-PC software (both Med Associates, St. Albans, Vermont).

Nicotine Self-Administration. We performed this phase over a period of 14 weeks; and 1-hour sessions were conducted from Monday through Friday. Before the start of each session, we placed the monkeys into the Plexiglas chairs and restrained them in the seated position by waist locks. We first trained the monkeys to lever press under a fixed-ratio schedule (FR10, timeout 60 sec) of intravenous nicotine (30 μ g/kg per injection) reinforcement. After flushing the catheters with 1 mL physiologic saline, we connected them to a motor-driven syringe. At the start of each session, the white house-light was turned off, and the green stimulus light was turned on; 10 lever presses turned off the green light and produced 2-sec amber light paired with nicotine injection (.2 mL). During the 60-sec timeout period, the chamber was dark, and lever presses had no programmed consequences. When responses showed $<15\%$ variability for at least five consecutive sessions, we tested the effect of AZD8529 pretreatment (.03 mg/kg, .3 mg/kg, 1 mg/kg, 3 mg/kg, and 10 mg/kg, intramuscular injection [i.m.], 3 hours before the session) on nicotine self-administration for three sessions; we compared these data with three consecutive sessions of vehicle pretreatment immediately preceding each test session. The 3-hour pretreatment time is based on AstraZeneca time to maximum concentration pharmacokinetic studies (data not shown).

Reinstatement of Nicotine Seeking. We performed this phase of the study over a period of 9 weeks. We first tested the monkeys for nicotine priming-induced reinstatement after extinction of the drug-reinforced responding. We then

retrained them to self-administer nicotine over 5-10 sessions and tested them for cue-induced reinstatement after extinction of the drug-reinforced responding. We tested AZD8529 doses of ≤ 3 mg/kg because 3 mg/kg was the highest effective dose that reduced nicotine but not food self-administration.

Nicotine Priming-Induced Reinstatement. We performed tests for nicotine priming-induced reinstatement after the monkeys underwent daily extinction sessions during which lever presses led to saline infusions plus the visual cues previously paired with nicotine infusions, but not nicotine. We gave a noncontingent saline injection before each extinction session as a vehicle control for the nicotine-priming injections. After at least two extinction sessions, when responding had reached a low, stable level, we determined the effect of pretreatment with AZD8529 (.3 mg/kg, 1 mg/kg, or 3 mg/kg, i.m.) or its vehicle on nicotine-induced (.1 mg/kg intravenous injection) reinstatement. We gave the nicotine priming injections immediately before the start of the test sessions. During testing, lever presses (FR10) continued to produce only saline injections and the discrete cues. We also tested the effect of 3 mg/kg of AZD8529 on saline priming to determine whether AZD8529 alone would affect nicotine seeking after extinction.

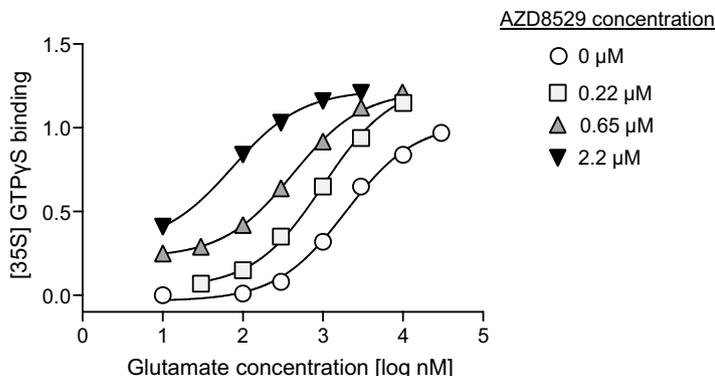
Cue-Induced Reinstatement. After the completion of nicotine priming tests, we retrained the monkeys to self-administer nicotine for 5-10 sessions. We then gave them three extinction sessions during which lever presses had no reinforced consequences (neither nicotine nor cues were available); additionally, we did not inject monkeys with saline priming before these sessions. After extinction, we determined the effect of pretreatment with AZD8529 (.3 mg/kg, 1 mg/kg, or 3 mg/kg, i.m.) or its vehicle on cue-induced reinstatement. During testing, lever presses (FR10) produced the intravenous saline infusions and visual cue presentations. We also determined the effect of 3 mg/kg of AZD8529 on extinction responding in the absence of the cue. Each cue-induced reinstatement test was followed by one or two extinction sessions.

Food Self-Administration. We determined the effect of AZD8529 in a separate group of monkeys that self-administered 190-mg food pellets under reinforcement schedule conditions identical to the conditions we used with nicotine (FR10, timeout 60 sec). We restricted food intake to maintain monkeys' weights at a level that facilitates food-reinforced responding (no more than 10% below free-feeding weight). The number of reinforcers delivered per session and rates of responding in this group were very similar to the nicotine group. We injected each dose of AZD8529 (3 mg/kg, 10 mg/kg, and 30 mg/kg, i.m.) for three consecutive sessions, which was preceded by three sessions with vehicle injections before the sessions.

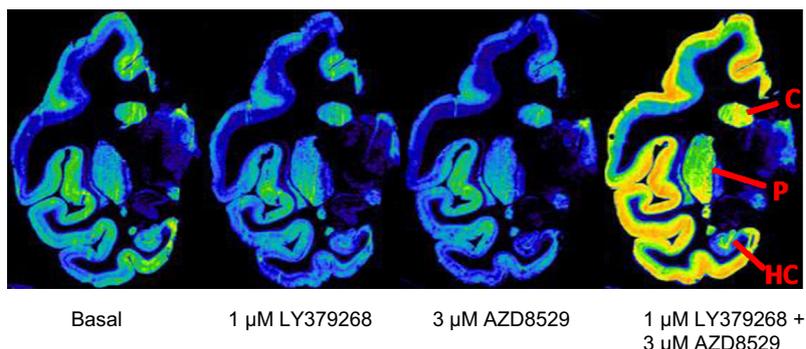
AZD8529 Plasma Levels in Squirrel Monkeys

To determine whether plasma levels during the behavioral experiments reach levels that are well tolerated in humans

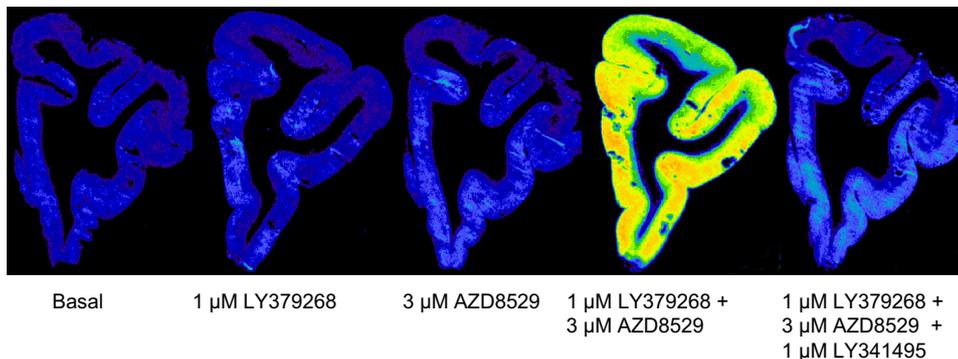
A. [³⁵S]GTPγS binding in human mGluR2-CHO membranes



B. [³⁵S]GTPγS autoradiography in cynomolgus monkey brain slices



C. [³⁵S]GTPγS autoradiography in cynomolgus monkey brain slices (level of mPFC)



(per AstraZeneca company information), we injected three squirrel monkeys with AZD8529 (1 mg/kg, i.m.) and 3 hours later collected venous blood samples (~1.5 ml) from the femoral vein under light ketamine (10 mg/kg, i.m.) anesthesia. We rapidly mixed the blood samples and immediately cooled them on ice until centrifugation. Plasma was prepared by centrifugation at 4°C for 10 min at 1500 g within 30 min of blood sampling. We separated the plasma and transferred it to two 2-mL microcentrifuge tubes. We stored the plasma samples at -80°C. We shipped the samples on dry ice to AstraZeneca where AZD8529 levels were measured using a standardized liquid chromatography coupled to tandem mass spectrometry method.

Figure 1. Activity of AZD8529 at metabotropic glutamate receptors using functional assays. **(A)** Effect of increasing concentrations of AZD8529 on [³⁵S]GTPγS binding to human mGluR2 expressed in Chinese hamster ovary (CHO) cells in the presence of increasing concentrations of the agonist L-glutamate. Data are from a representative experiment that was repeated three times. AZD8529 potentiated the effects of glutamate at mGluR2 with an apparent potency (EC₅₀) of 195 ± 62 nmol/L and a maximal efficacy (E_{max}) of 110% ± 11%. **(B)** Effect of AZD8529 on binding of [³⁵S]GTPγS to cynomolgus monkey brain slices revealed by quantitative autoradiography. Representative digitized autoradiograms are shown from a single experiment repeated three times. Basal, [³⁵S]GTPγS binding in the absence of added drug; agonist alone, [³⁵S]GTPγS binding in the presence of a suboptimal concentration of mGluR2/mGluR3 agonist 1 μmol/L LY379268; modulator alone, [³⁵S]GTPγS binding in the presence of 3 μmol/L AZD8529 with no agonist addition; modulator + agonist, [³⁵S]GTPγS binding in the presence of both agonist 1 μmol/L LY379268 and modulator 3 μmol/L AZD8529. The caudate nucleus (C), putamen (P), and hippocampus (HC) are annotated on the right panel. **(C)** Potentiation of agonist-induced activation of mGluR2 by AZD8529 was reversed by the mGluR2/mGluR3 antagonist LY341495. Representative autoradiograms are shown from the level of medial prefrontal cortex, and the combination of modulator + agonist + antagonist is shown in the right panel: [³⁵S]GTPγS binding in the presence of agonist 1 μmol/L LY379268, modulator 3 μmol/L AZD8529, and antagonist 1 μmol/L LY341495.

In Vivo Microdialysis in Rats

The general procedure was described previously (36). We performed microdialysis in Sprague-Dawley rats 20–24 hours after implantation of probes aimed at the accumbens shell (2.0 mm anterior, 1.1 mm lateral from bregma, and 8.0 mm below the dura mater) (37). We collected samples (20 μL) every 20 min (perfusion rate 1 μL/min) and immediately analyzed dopamine levels by high-performance liquid chromatography coupled to electrochemical detection. We injected the test drugs or their vehicle after observing stable dopamine levels (<15% variation) in three consecutive samples. We injected vehicle or AZD8529 (10 mg/kg or 30

mg/kg intraperitoneal injection) 2 hours before injecting vehicle or nicotine (4 mg/kg subcutaneous injection). We collected dialysate samples for 2 hours after nicotine injections. We based the AZD8529 doses on previous unpublished work of AstraZeneca in rat behavioral models and a recent study on the effect of the drug on “incubation” of methamphetamine craving in rats (38).

Drugs

We dissolved nicotine ((-)-nicotine hydrogen tartrate; Sigma-Aldrich, St Louis, Missouri) in saline and adjusted the pH of the solution to 7.0 by diluted sodium hydroxide. We dissolved AZD8529 (7-methyl-5-(3-piperazin-1-ylmethyl-[1,2,4]oxadiazol-5-yl)-2-(4-trifluoromethoxybenzyl)-2,3-dihydroisindol-1-one; AstraZeneca) in sterile water (Hospira, Lake Forest, Illinois). We express all nicotine and AZD8529 doses as free-base.

Statistical Analysis

We recorded the number of lever presses and number of injections per sessions. We calculated response rates based on available session time for responding (i.e., timeout time was subtracted). We also recorded timeout responses. We analyzed the nicotine or food self-administration data with repeated measures analysis of variance (SigmaStat, Systat Software, San Jose, California), using the within-subjects factors of AZD8529 dose and treatment session (session 1, 2, 3). We analyzed the nicotine priming-induced and cue-induced reinstatement data with repeated measures analysis of variance, using the within-subjects factor of AZD8529 dose. We expressed the microdialysis data as a percentage of basal dopamine values; basal values were the mean of three consecutive samples (differing from each other by <15%) taken immediately before the first injection of AZD8529 or vehicle. We analyzed these data using repeated measures analysis of variance. We followed up on significant main or interaction effects ($p < .05$) using Tukey post hoc tests.

RESULTS

AZD8529 Potentiation of mGluR2 Receptor Function

We assessed the effect of AZD8529 at the human mGluR2 receptor by measuring the potentiation of [³⁵S]GTPγS binding in the presence of increasing concentrations of exogenously applied agonist (L-glutamate). AZD8529 potentiated the effects of glutamate at mGluR2 with an EC₅₀ of 195 ± 62 nmol/L and an E_{max} of 110% ± 11% (Figure 1A). To assess the selectivity of AZD8529 against the family of mGluRs, we used fluorescence-based assays. AZD8529 potentiated mGluR2 activity with an EC₅₀ of 285 ± 20 nmol/L and did not produce any positive allosteric modulator responses at 20–25 μmol/L on the mGluR1, mGluR3, mGluR4, mGluR5, mGluR6, mGluR7, and mGluR8 subtypes (Table 1). In addition, at 25 μmol/L, AZD8529 did not elicit antagonist responses on mGluRs. When AZD8529 (10 μmol/L) was studied in a broad receptor screen (Table 2), we observed >50% inhibition of ligand binding at adenosine A3 receptors (51% inhibition) and the norepinephrine transporter (IC₅₀ = 4.73 μmol/L).

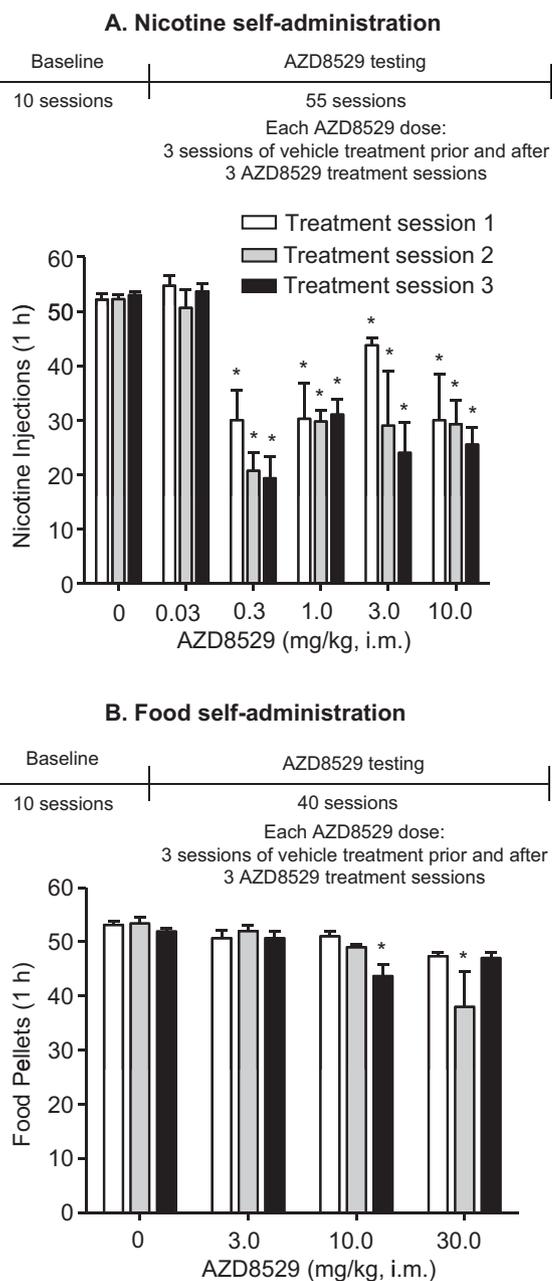


Figure 2. Effect of AZD8529 on nicotine and food self-administration in squirrel monkeys. Mean ± SEM of nicotine injections (30 μg/kg) (A) or food pellets (B) per 1-hour session after pretreatment (3 hours) with vehicle or AZD8529 (.03–30 mg/kg) for three consecutive sessions. Also shown is the experimental timeline (see Methods and Materials). Different from the mean of three sessions of vehicle (0 mg/kg) treatment, * $p < .05$, $n = 3–4$. i.m., intramuscular (injection).

We also determined the ability of AZD8529 to potentiate agonist-induced activation of mGluR2 in the primate brain by using [³⁵S]GTPγS autoradiography on slices prepared from a cynomolgus monkey brain (Figure 1B). This method provides measures of the efficacy of allosteric modulators at mGluR2 and anatomic localization for the measured activity. AZD8529 (3 μmol/L) significantly potentiated LY379268 (1 μmol/L) activation of the

Table 1. Testing of Selectivity of AZD8529 at mGluRs

Receptor	Assay	Agonist	Agonist Concentration	AZD8529 Maximum Concentration	Effect
mGluR2	Agonist			20 μ mol/L	NSE
	Positive modulator	DCG-IV	.02 μ mol/L	20 μ mol/L	EC ₅₀ = 285 \pm 20 nmol/L, E _{max} = 59.9% \pm 14%
	Antagonist	DCG-IV	.2 μ mol/L	20 μ mol/L	NSE
mGluR3	Agonist			NT	NSE
	Positive modulator	DCG-IV	.02 μ mol/L	25 μ mol/L	NSE
	Antagonist	DCG-IV	.2 μ mol/L	25 μ mol/L	NSE
mGluR1	Agonist			20 μ mol/L	NSE
	Positive modulator	3,5,DHPG	.2 μ mol/L	20 μ mol/L	NSE
	Antagonist	3,5,DHPG	1.0 μ mol/L	20 μ mol/L	NSE
mGluR5	Agonist			25 μ mol/L	NSE
	Positive modulator	3,5,DHPG	.2 μ mol/L	25 μ mol/L	NSE
	Antagonist	3,5,DHPG	1.0 μ mol/L	25 μ mol/L	NSE
mGluR6	Agonist			20 μ mol/L	NSE
	Positive modulator	L-AP4	.004 μ mol/L	20 μ mol/L	NSE
	Antagonist	L-AP4	.1 μ mol/L	20 μ mol/L	NSE
mGluR7	Agonist			20 μ mol/L	NSE
	Positive modulator	L-AP4	26 μ mol/L	20 μ mol/L	NSE
	Antagonist	L-AP4	200 μ mol/L	20 μ mol/L	NSE
mGluR4	Agonist			NT	NSE
	Positive modulator	DL-AP4	.06 μ mol/L	25 μ mol/L	NSE
	Antagonist	DL-AP4	.4 μ mol/L	25 μ mol/L	NSE
mGluR8	Agonist			NT	NSE
	Positive modulator	DL-AP4	.06 μ mol/L	25 μ mol/L	NSE
	Antagonist	DL-AP4	.4 μ mol/L	25 μ mol/L	NSE

The selectivity of AZD8529 at mGluRs was tested in fluorescence-based binding assays using HEK 293 cell lines expressing human chimeric fusion constructs hmGluR2/hCaR*, hmGluR1/hCaR*, hmGluR3/hCaR*, hmGluR4/hCaR*, hmGluR5/hCaR*, hmGluR6/hCaR*, hmGluR7/hCaR*, and hmGluR8/hCaR*.

DCG-IV, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; 3,5,DHPG, (S)-3,5-dihydroxyphenylglycine; DL-AP4, DL-2-amino-4-phosphonobutyric acid; EC₅₀, half maximal effective concentration; E_{max}, maximal efficacy; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; mGluRs, metabotropic glutamate receptors; NSE, nonsignificant effect; NT, not tested.

[³⁵S]GTP γ S signal in the monkey brain compared with LY379268 alone. We found that AZD8529 potentiated the [³⁵S]GTP γ S signal in the cortex, hippocampus, and striatum. The potentiation of LY379268 activation of the [³⁵S]GTP γ S signal by AZD8529 was reversed by the mGluR2/mGluR3 antagonist LY341495 (1 μ mol/L) (Figure 1C, medial prefrontal cortex level shown).

Effect of AZD8529 on Nicotine Self-Administration

Under baseline conditions, nicotine (30 μ g/kg per injection) maintained high rates of responding, with significantly more injections per session (mean \pm SEM 52.1 \pm 1.1) and responses/sec (1.3 \pm .2) than when saline was substituted for nicotine (5.2 \pm .3 injections per session and .02 \pm .01 responses/sec). AZD8529 at doses of .3 mg/kg, 1 mg/kg, 3 mg/kg, and 10 mg/kg (but not .03 mg/kg) decreased nicotine self-administration (Figure 2A). The statistical analysis showed a significant effect of AZD8529 dose on number of infusions [$F_{4,24} = 16.2, p < .01$] and response rate [$F_{4,24} = 11.8, p < .01$] (Table 3). The dose \times treatment session interaction was not significant ($p > .1$), and there was no difference among treatment sessions ($p > .1$). The latter finding indicates that tolerance did not develop to the effects of AZD8529 on nicotine self-administration over repeated testing. Nicotine self-administration behavior rapidly returned to higher levels when treatment with AZD8529 was discontinued.

Effect of AZD8529 of Food-Maintained Operant Responding

The monkeys trained under the FR10 schedule earned 52.8 \pm .8 pellets/session and lever pressed at a rate of 1.5 \pm .2 responses/sec (Figure 2B). AZD8529 at doses of 10 mg/kg and 30 mg/kg (but not 3 mg/kg, the highest dose in the reinstatement experiments) decreased the number of pellets earned (AZD8529 dose \times treatment session interaction [$F_{6,12} = 3.1, p < .01$]) and response rate (AZD8529 dose \times treatment session interaction [$F_{6,12} = 3.3, p < .01$]) (Table 3). This interaction is due to the different effects of the 10 mg/kg and 30 mg/kg doses on food self-administration over repeated testing.

Effect of AZD8529 on Reinstatement of Nicotine Seeking

Nicotine Priming-Induced Reinstatement. Nicotine priming injections (.1 mg/kg intravenous injection) reinstated nicotine seeking (lever presses, 520.0 \pm 8.4 after nicotine priming vs. 42.9 \pm 7.6 after saline priming; response rate, 1.2 \pm .17 responses/sec after nicotine priming vs. .02 \pm .01 after saline priming) (Figure 3A and Table 4). Pretreatment with AZD8529 decreased nicotine-induced reinstatement in a dose-dependent manner (lever presses [$F_{5,15} = 39.6, p < .01$]; response

Table 2. Effects of AZD8529 at 10 μmol/L in a Broad Receptor Screen Using Radioligand Binding Assays

Target	Source	% Inhibition at 10 μmol/L	IC ₅₀ μmol/L ^a
Adenosine A ₁	Human rCHO cells	-7	NT
Adenosine A _{2A}	Human rHEK 293 cells	8	NT
Adenosine A ₃	Human rCHO-K1 cells	51	NT
Adrenergic α _{1A}	Rat submaxillary gland	17	NT
Adrenergic α _{1B}	Rat liver	13	NT
Adrenergic α _{1D}	Human rHEK 293 cells	12	NT
Adrenergic α _{2A}	Human rSf9 insect cells	41	NT
Adrenergic α _{2C}	Human rSf9 insect cells	34	NT
Adrenergic β ₁	Human rRex 16 cells	10	NT
Adrenergic β ₂	Human rCHO cells	7	NT
Adrenergic β ₃	Human rHEK 293 cells	4	NT
Cannabinoid CB ₁	Human rHEK 293 cells	11	NT
Cannabinoid CB ₁	Human rCHO-K1 cells	26	NT
Dopamine D ₁	Human rCHO cells	7	NT
Dopamine D ₂	Human rCHO cells	-4	NT
Dopamine D ₃	Human rCHO cells	14	NT
Dopamine D ₄	Human rCHO-K1 cells	-8	NT
Dopamine D ₅	Human rCHO cells	17	NT
GABA _A (Agonist)	Rat brain (no cerebellum)	1	NT
GABA _A (BDZ)	Rat brain (no cerebellum)	-15	NT
GABA _{B1A}	Human rCHO cells	16	NT
GABA _{B1B}	Human rCHO cells	-6	NT
Glutamate, AMPA	Rat cerebral cortex	-19	NT
Glutamate, Kainate	Rat brain (no cerebellum)	4	NT
Glutamate, NMDA glycine	Rat cerebral cortex	-11	NT
Glutamate, NMDA PCP	Rat cerebral cortex	10	NT
Glutamate, NMDA Polyamine	Rat cerebral cortex	-14	NT
Glycine, Strychnine Sensitive	Rat spinal cord	8	NT
Histamine H ₁	Human rCHO cells	29	NT
Histamine H ₂	Human rCHO-K1 cells	27	NT
Histamine H ₃	Human rCHO-K1 cells	11	NT
Muscarinic M ₁	Human rCHO cells	33	NT
Muscarinic M ₂	Human rCHO cells	25	NT
Muscarinic M ₃	Human rCHO cells	7	NT
Muscarinic M ₄	Human rCHO cells	21	NT
Muscarinic M ₅	Human rCHO cells	38	NT
Nicotinic α ₁	Human RD cells	9	NT
Nicotinic α ₇	Rat brain (no cerebellum)	4	NT
Opiate delta (δ)	Human rCHO cells	45	NT
Opiate kappa (κ)	Human rHEK 293 cells	6	NT
Opiate mu (μ)	Human rCHO-K1 cells	7	NT
Orphanin ORL1	Human rHEK 293 cells	-7	NT
Serotonin 5-HT _{1A}	Human rCHO cells	6	NT
Serotonin 5-HT _{1B}	Rat cerebral cortex	16	NT
Serotonin 5-HT _{2B}	Human rCHO-K1 cells	19	NT
Serotonin 5-HT _{2C}	Human rCHO-K1 cells	19	NT
Serotonin 5-HT ₃	Human rHEK 293 cells	9	NT
Serotonin 5-HT _{5A}	Human rCHO-K1 cells	-17	NT

Table 2. Continued

Target	Source	% Inhibition at 10 μmol/L	IC ₅₀ μmol/L ^a
Serotonin 5-HT ₆	Human rHeLa cells	13	NT
Dopamine transporter	Human rCHO-K1 cells	44	NT
GABA transporter	Rat cerebral cortex	15	NT
Norepinephrine transporter	Human rMDCK cells	81	4.73
Serotonin transporter	Human rHEK 293 cells	5	NT

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CHO, Chinese hamster ovary; GABA, gamma-aminobutyric acid; 5-HT, 5-hydroxytryptamine; IC₅₀, half maximal inhibitory concentration; NMDA, N-methyl-D-aspartate; NT, not tested; ORL, opioid receptor like; PCP, phencyclidine; r, recombinant (e.g., rCHO cells, recombinant CHO cells). ^aIC₅₀ value was determined by a nonlinear, least squares regression analysis.

rate [$F_{5,15} = 41.9, p < .01$] (Figure 3A and Table 4). When the 3 mg/kg dose of AZD8529 was injected before vehicle priming, it did not reinstate extinguished drug seeking ($p > .1$).

Cue-Induced Reinstatement. When responding no longer produced nicotine or the interoceptive cues produced by intravenous injection or the visual cues that were previously associated with nicotine, response rates of monkeys decreased to very low levels (lever presses, 50.0 ± 13.4 ; response rate, $.02 \pm .01$ responses/sec) (Figure 3B and Table 4). Reintroduction of the response-dependent, nicotine-associated cues (injection-related and visual) reinstated nicotine seeking (lever presses, 525.0 ± 6.4 ; response rate, $1.3 \pm .14$ responses/sec). Pretreatment with AZD8529 (.3 mg/kg, 1.0 mg/kg, or 3 mg/kg) decreased cue-induced reinstatement (lever presses [$F_{5,14} = 35.6, p < .01$]; responses rate [$F_{5,14} = 56.8, p < .01$]). The highest AZD8529 dose (3 mg/kg) had no effect on baseline extinction responding without the cues ($p > .1$).

Plasma Concentrations of AZD8529

In a group of squirrel monkeys ($n = 3$), the plasma concentration of AZD8529 3 hours (the pretreatment time in the self-administration and reinstatement experiments) after drug (1 mg/kg) injections was 112 ± 17 nmol/L.

Effect of AZD8529 on Nicotine-Induced Dopamine Release in the Rat Accumbens Shell

We determined the effect of systemic AZD8529 injections on nicotine-induced elevations of extracellular dopamine levels in accumbens shell of freely moving rats. Nicotine (.4 mg/kg, subcutaneous injection) increased extracellular dopamine, and this effect was decreased by AZD8529 30 mg/kg, but not 10 mg/kg (AZD8529 dose \times time interaction [$F_{34,170} = 2.24, p < .001$]) (Figure S1 in Supplement 1). When given alone, AZD8529 (10 mg/kg or 30 mg/kg) had no effect on dopamine levels (Figure S1 in Supplement 1).

DISCUSSION

AZD8529, a potent and highly selective positive allosteric modulator of mGluR2, decreased nicotine self-administration

Table 3. Effect of AZD8529 on Response Rate During Nicotine or Food Self-Administration Sessions (n = 4)

AZD8529 Dose (mg/kg, i.m.)	Nicotine Self-Administration, Responses/Sec (Mean ± SEM)	Food Self-Administration, Responses/Sec (Mean ± SEM)
0	1.39 ± .17	1.47 ± .17
.03	1.66 ± .45 ^a	
.3	.13 ± .04 ^a	
1	.19 ± .02 ^a	
3	.31 ± .07 ^a	1.08 ± .15
10	.19 ± .04 ^a	.74 ± .02 ^a
30		.55 ± .03 ^a

Data are means of three sessions of vehicle or AZD8529 treatment.

i.m., intramuscular (injection).

^aSignificantly different from vehicle pretreatment (0 mg/kg).

and nicotine priming-induced and cue-induced reinstatement in monkeys and caused these effects at doses 3-fold to 10-fold lower than the doses that decreased food self-administration. The finding that a PAM for mGluR2 decreased nicotine self-administration is consistent with previous findings with the orthosteric agonist LY379268 in rats (20). However, the allosteric modulator AZD8529 exhibited a more promising therapeutic profile, having no effect on food-maintained responding at doses (.3–3 mg/kg) that had robust effects on nicotine self-administration and reinstatement of nicotine seeking. We did not observe tolerance to the effect of AZD8529 after repeated administration (Figure 2A). Finally, the selective effect of the lower doses of AZD8529 for nicotine self-administration and reinstatement versus food self-administration indicates that it is unlikely that motor deficits or other nonspecific behavioral effects of AZD8529 mediate its effect on nicotine-seeking behaviors. We also found that AZD8529 decreased nicotine-induced dopamine release in the rat accumbens shell, an important brain area for drug reward (39). The relevance of these rat results to the effect of AZD8529 on nicotine self-administration and reinstatement in monkeys is a subject for future research.

Our data indicate that AZD8529 is selective for mGluR2 over a wide range of targets (Tables 1 and 2) with very high doses of AZD8529 causing only partial inhibition of radioligand binding at adenosine A3 receptor and the norepinephrine transporter. AZD8529 was also well tolerated in human studies (30) at plasma concentrations that were achieved in monkeys following an effective drug dose in the present study. Our recombinant and native receptor [³⁵S]GTPγS assay results showed that AZD8529 selectively potentiates agonist-induced activation of mGluR2 in a cell culture containing the human mGluR2 and in the primate brain. This potentiation was reversed by a selective antagonist at mGluR2/mGluR3 in the primate brain. Because AZD8529 is inactive at mGluR3 (Table 1), these data indicate that the effects of AZD8529 are mediated by mGluR2.

The findings that AZD8529 blocked the effects of nicotine re-exposure and nicotine-associated cues may provide information about the nature of the effect of AZD8529 on nicotine

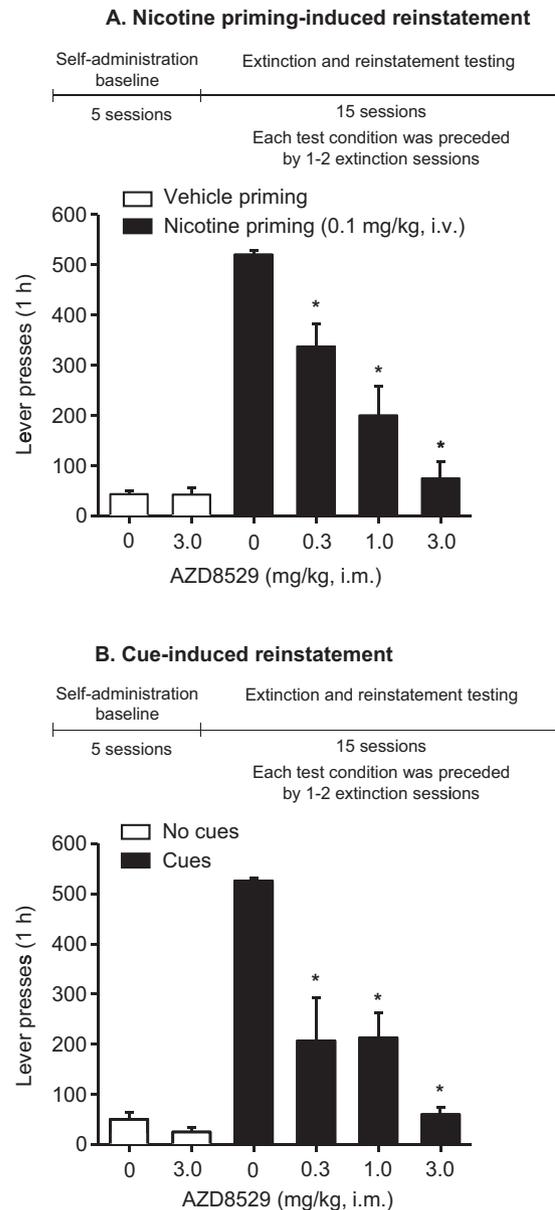


Figure 3. AZD8529 decreased nicotine priming-induced and cue-induced reinstatement in squirrel monkeys. Mean ± SEM number of nonreinforced lever presses during the tests for nicotine priming-induced reinstatement (A) or cue-induced reinstatement (B). Also shown is the experimental timeline (see Methods and Materials). During the extinction sessions before the tests for nicotine priming-induced reinstatement, saline was substituted for nicotine, and lever presses led to visual cue presentations. During the extinction sessions before the tests for cue-induced reinstatement, intravenous injections and visual cues were discontinued. During the test sessions, AZD8529 (.3, 1, or 3 mg/kg) or vehicle was injected 3 hours before the vehicle or nicotine priming injections (.1 mg/kg i.v.) (A) or reintroduction of cues (B); lever presses (fixed-ratio schedule [FR10]) produced intravenous saline injections and the cues in both tests. “Vehicle priming + 0 mg/kg” or “No cues + 0 mg/kg” represents the mean ± SEM of lever presses of five extinction sessions before the test sessions. “Nicotine priming + 0 mg/kg” or “Cues + 0 mg/kg” represents mean ± SEM of lever presses from two tests. Different from “Vehicle priming + 0 mg/kg” condition (A) or “Cues + 0 mg/kg” condition (B), **p* < .05, *n* = 4. i.m., intramuscular (injection); i.v., intravenous (injection).

Table 4. Effect of AZD8529 on Response Rates During Extinction Sessions and Nicotine Priming–Induced or Cue-Induced Reinstatement Tests (*n* = 4)

AZD8529 Dose (mg/kg, i.m.)	Nicotine Priming–Induced Reinstatement, Responses/Sec (Mean ± SEM)	Cue-Induced Reinstatement, Responses/Sec (Mean ± SEM)
0 + “Vehicle Priming” or “No Cues”	.02 ± .01	.02 ± .01
3.0 + “Vehicle Priming” or “No Cues”	.02 ± .01	.01 ± .01
0 + “Nicotine Priming” or “Cues”	1.20 ± .17	1.33 ± .14
.3 + “Nicotine Priming” or “Cues”	.25 ± .06	.13 ± .08
1.0 + “Nicotine Priming” or “Cues”	.11 ± .06	.11 ± .03
3.0 + “Nicotine Priming” or “Cues”	.03 ± .01	.02 ± .01

i.m., intramuscular (injection).

self-administration. Drug self-administration involves the direct reinforcing effects of the drug and the conditioned reinforcing effects of drug-associated cues (40). In particular, it has been shown in the case of nicotine self-administration that drug self-administration also involves nicotine-induced potentiation of the weak reinforcing effects of cues typically associated with nicotine administration (41–43). There is evidence that nicotine addiction depends on the behavioral and psychological effects of internal (interoceptive) and external (exteroceptive) cues associated with cigarette smoking (5).

Using a reinstatement model (44), we found that AZD8529 decreased not only the effects of re-exposure to nicotine (nicotine priming) but also the effects of nicotine-associated cues, which can provoke nicotine craving and relapse in humans (45). These findings are important because relapse is the primary obstacle to successful nicotine addiction treatment (3,46). It has been shown that cue-induced craving can increase with longer abstinence duration in smokers, even as background craving and withdrawal symptoms subside (47). The ability to block the direct reinforcing effects of nicotine, nicotine’s priming effect on cue responding, and the effects of smoking-related cues could make AZD8529 (and related PAMs of mGluR2) more effective than drugs that block only the direct reinforcing effects of nicotine.

In addition to affecting reinstatement of nicotine seeking (20), orthosteric agonism of mGluR2/mGluR3 by LY379268 and related drugs has also been shown to decrease different forms of relapse induced by conditioned cues and contexts that were previously associated with the self-administration of alcohol (16), heroin (13,48), cocaine (12,15,17), and methamphetamine (18). To the degree that these results are mediated by mGluR2 (as suggested by the present findings with AZD8529 and previous findings with BINA and a related PAM of GluR2 (21,27)), these observations may have implications for relapse prevention across drug classes because of the well-established comorbidity between nicotine addiction and alcoholism (48,49) and addiction to other drugs such as tetrahydrocannabinol, methamphetamine, heroin, and cocaine (50–52).

In conclusion, AZD8529 represents a class of orally bioavailable compounds that selectively enhance presynaptic glutamate signaling by binding at an allosteric site of mGluR2s and may have a more desirable and selective therapeutic profile than currently available orthosteric agonists that non-selectively act on mGluR2 and mGluR3 subtypes. The results of the present study provide novel evidence for the efficacy of PAMs of mGluR2 drugs in nonhuman primate models of nicotine reinforcement and relapse. This drug class should be considered for nicotine addiction treatment and potentially treatment of other addictions because AZD8529 also was shown recently to decrease cue-induced methamphetamine seeking after prolonged forced or voluntary abstinence in a rat model of “incubation” of drug craving (38).

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by the National Institute on Drug Abuse Intramural Research Program and Division of Pharmacotherapies and Medical Consequences of Drug Abuse, National Institutes of Health, Department of Health and Human Services. AZD8529 was kindly provided by AstraZeneca.

We thank Drs. Jane Acri and Phil Skolnick for their valuable insights during the planning of the study and preparation of this manuscript. We thank Dr. Ira Baum and Philip White for their excellent veterinary assistance during the study.

We dedicate this study to our dear mentor, colleague, and friend, Dr. Steven R. Goldberg, who passed suddenly on November 25, 2014.

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Received Nov 13, 2014; revised Jan 27, 2015; accepted Jan 28, 2015.

Supplementary material cited in this article is available online at <http://dx.doi.org/10.1016/j.biopsych.2015.01.014>.

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