GZ-11608, a Vesicular Monoamine Transporter-2 Inhibitor, Decreases the Neurochemical and Behavioral Effects of Methamphetamine^[S]

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Received April 8, 2019; accepted August 1, 2019

ABSTRACT

Despite escalating methamphetamine use and high relapse rates, pharmacotherapeutics for methamphetamine use disorders are not available. Our iterative drug discovery program had found that *R-N*-(1,2-dihydroxypropyl)-2,6-*cis*-di-(4-methoxyphenethyl)piperidine hydrochloride (GZ-793A), a selective vesicular monoamine transporter-2 (VMAT2) inhibitor, specifically decreased methamphetamine's behavioral effects. However, GZ-793A inhibited human-ether-a-go-go-related gene (hERG) channels, suggesting cardiotoxicity and prohibiting clinical development. The current study determined if replacement of GZ-793A's piperidine ring with a phenylalkyl group to yield *S*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine (GZ-11608) diminished hERG interaction while retaining pharmacological efficacy. VMAT2 inhibition, target selectivity, and mechanism of GZ-11608-induced inhibition of methamphetamine-evoked vesicular dopamine release were determined. We used GZ-11608 doses that decreased methamphetamine-sensitized activity to evaluate the potential exacerbation of methamphetamine-induced dopaminergic neurotoxicity. GZ-11608-induced decreases in methamphetamine reinforcement and abuse liability were determined using self-administration, reinstatement, and substitution assays. Results show that GZ-11608 exhibited high affinity ($K_i = 25$ nM) and selectivity (92–1180-fold) for VMAT2 over nicotinic receptors, dopamine transporter, and hERG, suggesting low side-effects. GZ-11608 ($EC_{50} = 620$ nM) released vesicular dopamine 25-fold less potently than it inhibited VMAT2 dopamine uptake. GZ-11608 competitively inhibited methamphetamine-evoked vesicular

dopamine release (Schild regression slope = 0.9 ± 0.13). GZ-11608 decreased methamphetamine sensitization without altering striatal dopamine content or exacerbating methamphetamine-induced dopamine depletion, revealing efficacy without neurotoxicity. GZ-11608 exhibited linear pharmacokinetics and rapid brain penetration. GZ-11608 decreased methamphetamine self-administration, and this effect was not surmounted by increasing methamphetamine unit doses. GZ-11608 reduced cue- and methamphetamine-induced reinstatement, suggesting potential to prevent relapse. GZ-11608 neither served as a reinforcer nor substituted for methamphetamine, suggesting low abuse liability. Thus, GZ-11608, a potent and selective VMAT2 inhibitor, shows promise as a therapeutic for methamphetamine use disorder.

SIGNIFICANCE STATEMENT

GZ-11608 is a potent and selective vesicular monoamine transporter-2 inhibitor that decreases methamphetamine-induced dopamine release from isolated synaptic vesicles from brain dopaminergic neurons. Results from behavioral studies show that GZ-11608 specifically decreases methamphetamine-sensitized locomotor activity, methamphetamine self-administration, and reinstatement of methamphetamine-seeking behavior, without exhibiting abuse liability. Tolerance does not develop to the efficacy of GZ-11608 to decrease the behavioral effects of methamphetamine. In conclusion, GZ-11608 is an outstanding lead in our search for a therapeutic to treat methamphetamine use disorder.

Introduction

Methamphetamine use disorder (MUD) is characterized by a constellation of symptoms that includes relapse, continued use despite adverse consequences, and social impairment (American Psychiatric Association, 2013). From 2010 to 2016, United States law enforcement agency seizures of

methamphetamine increased 3.2-fold and overdose death rates increased 4.1-fold, indicative of escalating use (UNODC, United Nations Office on Drugs and Crime, 2014, 2018; <https://www.drugabuse.gov/related-topics/trends-statistics/overdose-death-rates>). Importantly, methamphetamine use has increased among opioid users as access to opioids has diminished; associations between these epidemics are being recognized (Ellis et al., 2018). In 2015, 135,000 Americans aged 12 and older sought treatment of MUD at publicly licensed facilities (DEA, U.S. Department of Justice Drug Enforcement Administration, 2018). Unfortunately, FDA-approved pharmacotherapeutics for MUD are not available.

This work was supported with funding from the National Institutes of Health Grants U01 DA013519, U01 DA043908, and UL1 TR001998.

<https://doi.org/10.1124/jpet.119.258699>.

[S] This article has supplemental material available at jpet.aspetjournals.org.

Fig. 1. Structures of lobeline, lobelane, GZ-793A, GZ-11610, and GZ-11608.

Substantial effort has been directed toward discovering a pharmacotherapeutic for MUD (see reviews: Ballester et al., 2017; Dwoskin et al., 2017; Reynolds et al., 2017). Methamphetamine redistributes dopamine from synaptic vesicles into the cytosol by interacting with the vesicular monoamine transporter-2 (VMAT2) and disrupting the vesicular pH gradient (Sulzer and Rayport, 1990; Sulzer et al., 1995; Dwoskin and Crooks, 2002). Also, methamphetamine reverses dopamine transporter (DAT) function, resulting in transport of dopamine from the cytosol into the extracellular compartment, ultimately mediating methamphetamine reward and abuse liability (Wise and Rompre, 1989; Volkow et al., 2017).

The current study extended our iterative drug discovery research targeting VMAT2 with the goal of obviating the neuropharmacological effects of methamphetamine. Initially, lobeline (Fig. 1), the major alkaloid in *Lobelia inflata*, was found to inhibit VMAT2 function ($K_i = 470$ nM) and reduce methamphetamine-evoked dopamine release from superfused striatal slices and from nucleus accumbens following in vivo microdialysis in rats (Miller et al., 2001; Nickell et al., 2010; Meyer et al., 2013). Lobeline also decreased intravenous methamphetamine self-administration in rats without the development of tolerance (Harrod et al., 2001). Furthermore, increasing the methamphetamine unit dose did not surmount the lobeline-induced decrease in responding for methamphetamine (Harrod et al., 2001). On the basis of these preclinical findings, lobeline was evaluated in phase 1b clinical trials and found to be safe in individuals actively

using methamphetamine (Jones, 2007). Owing to its physicochemical properties and pharmacokinetic profile, enthusiasm for its further clinical development diminished.

Lobelane (Fig. 1), a chemically defunctionalized lobeline analog, was identified from structure activity relationship (SAR) studies as exhibiting greater potency (10-fold) for VMAT2 and reduced affinity for $\alpha 4\beta 2$ and $\alpha 7$ nicotinic acetylcholine receptors, thereby augmenting VMAT2 selectivity (Miller et al., 2004; Zheng et al., 2005; Nickell et al., 2010). Lobelane decreased methamphetamine-induced hyperlocomotion and decreased methamphetamine self-administration; however, the development of tolerance limited its therapeutic utility (Neugebauer et al., 2007). Further SAR studies identified *R-N*-(1,2-dihydroxypropyl)-2,6-*cis*-di-(4-methoxyphenethyl)-piperidine hydrochloride (GZ-793A, Fig. 1), which contains an *N*-1,2-dihydroxypropyl in place of the *N*-methyl group in lobelane (Horton et al., 2011b). GZ-793A exhibited high affinity ($K_i = 29$ nM) for VMAT2, and inhibited methamphetamine-evoked dopamine release from striatal slices and synaptic vesicular preparations and from nucleus accumbens in in vivo microdialysis studies (Horton et al., 2011b, 2013; Meyer et al., 2013; Nickell et al., 2017). GZ-793A decreased methamphetamine self-administration and reinstatement of methamphetamine-seeking behavior (Alvers et al., 2012; Beckmann et al., 2012). Importantly, tolerance did not develop to the GZ-793A-induced decrease in methamphetamine self-administration (Beckmann et al., 2012). Unfortunately, GZ-793A interacted with

ABBREVIATIONS: ANOVA, analysis of variance; ATP-Mg²⁺, adenosine 5'-triphosphate magnesium salt; AUC, area under the curve; DA, dopamine; DAT, dopamine transporter; dofetilide, *N*-[4-[2-[methyl[2-[4-(methylsulfonamido)phenoxy]ethyl]amino]ethyl]phenyl]methanesulfonamide; EGTA, ethylene glycol tetraacetate; GBR-12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride; GZ-11608, *S*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine; GZ-11610, *R*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine; GZ-361B, 1,4-diphenethylpiperidine; GZ-793A, *R-N*-(1,2-dihydroxypropyl)-2,6-*cis*-di-(4-methoxyphenethyl)piperidine hydrochloride; hERG, human-ether-a-go-go-related gene; 5-HT, 5-hydroxytryptamine, serotonin; ISTD, internal standard; LC-MS, liquid chromatography-mass spectrometry; MLA, methyllycaconitine; MP, mobile phase; MUD, methamphetamine use disorder; nAChRs, nicotinic acetylcholine receptors; NIC, nicotine; PEI, polyethyleneimine; p.o., oral gavage; RO4-1284, (2*R*,3*S*,11*bS*)-2-ethyl-3-isobutyl-9,10-dimethoxy-2,2,4,6,7,11*b*-hexahydro-1*H*-pyrido[2,1- α]isoquinolin-2-ol; SERT, serotonin transporter; VMAT2, vesicular monoamine transporter-2.

the human-ether-a-go-go-related gene (hERG) channel, revealing potential for cardiotoxicity and precluding its further development (Abbott et al., 1999; Sanguinetti and Tristani-Firouzi, 2006; Nickell et al., 2017).

Expansion of the SAR studies focused on minimizing the hERG interaction and resulted in a new structural scaffold with a phenylalkyl moiety replacing the piperidine ring in GZ-793A (Lee et al., 2018). The enantiomerically pure lead analog *R*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine (GZ-11610, Fig. 1), was identified as having high affinity ($K_i = 8.7$ nM) and selectivity (1090-fold) for VMAT2 over hERG. GZ-11610 (oral gavage) specifically decreased methamphetamine-sensitized locomotor activity. Although limitations in efficacy and potency of GZ-11610 were noted, the new scaffold showed good potential for identifying a high-value lead compound.

The current study investigated the pharmacology of enantiomerically pure *S*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine (GZ-11608, Fig. 1). VMAT2 affinity, selectivity, and mechanism of inhibition of methamphetamine-evoked vesicular dopamine release were determined. The ability of GZ-11608 to exacerbate the methamphetamine-induced decrease in striatal dopamine content was evaluated *ex vivo*. Effects of GZ-11608 on methamphetamine-sensitized locomotor activity, methamphetamine self-administration, and reinstatement also were determined. Development of tolerance and the potential for methamphetamine to surmount the efficacy of GZ-11608 to decrease methamphetamine self-administration were determined. Furthermore, the abuse liability of GZ-11608 was evaluated by determining its ability to substitute for methamphetamine in the self-administration assay and by acquisition of intravenous GZ-11608 self-administration in drug-naïve rats. Pharmacokinetic studies determined if GZ-11608 clearance remained constant with dose and determined the extent of its penetration into the brain.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (body weight of 300–400 g during the conduct of experiments; Harlan, Indianapolis, IN) were individually housed for behavioral studies and housed under standard conditions for neurochemical and pharmacokinetic assays. Upon arrival, rats were given free access to food and water in their home cages, which were maintained at 24°C, 45% humidity, and 14/10-hour light/dark cycle. Rats acclimated to the environment for 1 week prior to initiation of experiments, and when used in behavioral experiments, rats were handled daily. During operant training, food in the home cage was limited to 5–10 g/day to maintain bodyweight at approx. 85%, and then free feeding continued once rats reached criteria for stable responding. Experiments were conducted during the light phase. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and in accordance with the 2011 National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chemicals. [^3H]Dopamine ([^3H]DA; dihydroxyphenylethylamine, 3,4-[7- ^3H]; specific activity, 24.8 Ci/mmol), [^3H]5-hydroxytryptamine ([^3H]5-HT; 5-hydroxytryptamine creatinine sulfate, 5-[1,2- ^3H][N]; specific activity, 29.5 Ci/mmol), [^3H]nicotine ([^3H]NIC; (L-)-[*N*-methyl- ^3H]; specific activity, 80.4 Ci/mmol), and Microscint 20 cocktail were obtained from PerkinElmer Inc. (Waltham, MA). [^3H]Dofetilide ([*N*-methyl- ^3H]; specific activity, 80 Ci/mmol) and [^3H]methyllycaconitine ([^3H]MLA; [1 α ,4*S*,6 β ,14 α ,16 β]-20-ethyl-1,6,14,16-tetramethoxy-4-[[[2-(3- ^3H)-[3- ^3H]-methyl-2,5-dioxo-1-pyrrolidinyl]benzoyl]oxy]methyl]-aconitane-7,8-diol; specific activity,

60 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). (+)-Methamphetamine hydrochloride, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12935), amitriptyline, cytosine, fluoxetine, *S*(-)-nicotine hydrogen tartrate salt, nomifensine, 1-octanesulfonic acid sodium salt, 3-(4-methoxyphenyl)propanoic acid, 5-hydroxytryptamine creatinine sulfate, adenosine 5'-triphosphate magnesium salt (ATP-Mg $^{2+}$), α -D-glucose, ammonium chloride, anhydrous sodium sulfate, ascorbate oxidase, catechol, celite, dichloromethane, diethyl ether, dimethylformamide, dopamine hydrochloride, ethyl acetate, EDTA, ethylene glycol tetraacetate (EGTA), hexane, hydrochloric acid, hydroxybenzotriazole, lithium aluminum hydride (LiAlH $_4$), Kolliphor EL, magnesium sulfate, methanesulfonyl chloride, methanol, methylene chloride, HEPES, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, pargyline hydrochloride, phenyllithium, polyethyleneimine (PEI), potassium hydroxide, potassium tartrate dibasic hemihydrate, *R*-propylene oxide, sodium azide, silica, sodium chloride, sodium hydroxide, sucrose, tetrahydrofuran, triethylamine, triphenylphosphine, tris[hydroxymethyl]aminomethane base, tetrahydrofuran, and tris[hydroxymethyl]aminomethane hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile, calcium chloride, citric acid, formic acid, hydrogen chloride, methanol, monopotassium phosphate, potassium chloride, sodium bicarbonate, and sodium hydroxide were obtained from Fisher Scientific Co. (Pittsburgh, PA). Ascorbic acid, 1% non-essential amino acids, and scintillation cocktail 3A70B were purchased from Analab-BHD Ltd. (Polle, UK), Thermo Fisher Scientific (Waltham, MA), and Research Products International Corp. (Mount Prospect, IL), respectively. Minimum essential medium, Hanks' Balanced Salt solution, and 10% fetal bovine serum were obtained from Gibco (Grand Island, NY). (2*R*,3*S*,11*bS*)-2-Ethyl-3-isobutyl-9,10-dimethoxy-2,2,4,6,7,11*b*-hexahydro-1*H*-pyrido[2,1-*a*]isoquinolin-2-ol (RO4-1284) was a generous gift from Hoffmann-LaRoche Inc. (Nutley, NJ).

GZ-11608 Synthesis. To a solution of phenyllithium (50 ml, 1.8 M in dibutyl ether) in tetrahydrofuran (50 ml) was added *R*-propylene oxide (5 g) dropwise at -78°C . The resulting mixture was stirred at -78°C for 1 hour before being warmed to room temperature and stirred overnight. The reaction was quenched by addition of saturated aqueous ammonium chloride solution (50 ml). The aqueous phase was extracted with ethyl acetate (3 $\times$ 50 ml) and the combined organic layers were washed with brine (100 ml), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was chromatographed on silica (hexanes/ethyl acetate 10:1 to 3:1) to afford *R*-1-phenylpropan-2-ol (10.4 g) as a colorless oil. To a solution of *R*-1-phenylpropan-2-ol (4.16 g, 30.5 mmol) and triethylamine (7.72 g, 10.64 ml, 76.27 mmol) in dichloromethane (100 ml) was added methane sulfonyl chloride (4.54 g, 3.07 ml, 39.65 mmol) dropwise at 0°C . The resulting mixture was stirred at 0°C for 15 minutes. Dichloromethane (100 ml) was added to the mixture, which was then washed with water (2 $\times$ 150 ml). The organic layer was separated, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The resulting light-yellow oil was mixed with sodium azide (5.95 g, 91.5 mmol) in dimethylformamide (40 ml) and the mixture heated at 55°C for 3 hours. The reaction mixture was diluted with diethyl ether (150 ml) and washed with water (2 $\times$ 100 ml) and brine (100 ml). The organic layer was separated, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was chromatographed on silica (hexanes/ethyl acetate 50:1 to 20:1) to afford *S*-(2-azidopropyl)benzene (4.38 g) as a colorless oil. To a solution of *S*-(2-azidopropyl)benzene (4.0 g, 24.81 mmol) in tetrahydrofuran (90 ml) and water (10 ml) was added triphenylphosphine (9.11 g, 34.74 mmol) at room temperature. The resulting mixture was stirred for 18 hours and water (50 ml) was added. Hydrochloric acid (1.0 M) was then added to the mixture to obtain a pH 1.0, and the aqueous phase was extracted with diethyl ether (3 $\times$ 100 ml) and dichloromethane (2 $\times$ 100 ml). Aqueous sodium hydroxide (15% w/v) was added dropwise

to the aqueous phase to adjust the pH to 11 and the solution extracted with dichloromethane (5×60 ml). The combined, separated organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to afford *S*-1-phenylpropan-2-amine as a colorless oil. The crude amino product (3.03 g, 22.41 mmol) was mixed with 3-(4-methoxyphenyl)-propanoic acid (4.44 g, 24.65 mmol), and hydroxybenzotriazole (3.63 g, 26.89 mmol) in dichloromethane (60 ml) at room temperature. Triethylamine (5.67 g, 56.03 mmol) was added followed by *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (5.15 g, 26.89 mmol). The resulting mixture was stirred overnight. The reaction mixture was diluted with dichloromethane (100 ml) and washed with water (3×50 ml) and brine (50 ml). The organic layer was separated and dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was chromatographed on silica (dichloromethane/ethyl acetate 10:1) to afford *S*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propanamide (6.18 g) as a white solid. To a solution of *S*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propanamide (420 g, 1.41 mmol) in tetrahydrofuran (7 ml), LiAlH_4 (5.6 ml, 1.0 M in tetrahydrofuran) was added dropwise at 0°C . The resulting mixture was heated at reflux for 3 hours before being cooled to 0°C . The reaction was quenched by careful addition of water (0.21 ml), followed by 15% w/v aqueous sodium hydroxide (0.21 ml) and water (0.63 ml). The resulting milky suspension was warmed to room temperature, anhydrous magnesium sulfate was added, and the mixture was stirred for 2 hours, filtered through a pad of Celite, and rinsed with ethyl acetate. The combined filtrates were concentrated under vacuum, and the crude product was chromatographed on silica (methylene chloride/methanol, 30:1 to 10:1) to afford GZ-11608 (380 mg, 95%) as a white solid with low melting point: ^1H NMR (400 MHz, CDCl_3) δ 7.15–7.34 (m, 5H), 7.01 (d, $J = 8.4$ Hz, 2H), 6.80 (dd, $J = 8.4$, 2.0 Hz, 2H), 3.77 (s, 3H), 2.87 (m, 1H), 2.47–2.77 (m, 6H), 1.73 (m, 2H), 1.05 ppm (d, $J = 6.4$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ 157.9, 139.7, 134.2, 129.5, 129.4, 128.6, 126.4, 113.9, 55.4, 54.8, 46.8, 43.8, 32.8, 32.1, 20.4 ppm; MS (EI) m/z 282.2 $[\text{M}-1]^+$; purity: >97% liquid chromatography–mass spectrometry (LC-MS). GZ-11608 was converted to hydrochloride salt by treating with HCl in ether (2 M). The salt form was used for all the experiments.

Vesicular [^3H]DA Uptake. To obtain the affinity (K_i value) of GZ-11608 for VMAT2, the ability of GZ-11608 to inhibit [^3H]DA uptake into isolated synaptic vesicles in a concentration-dependent manner was determined, as previously described (Teng et al., 1997). Nonspecific uptake of [^3H]DA was determined in the presence of RO4-1284 (10 μM). Briefly, striata from individual rats were homogenized in 14 ml of ice-cold 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4) with 10 up-and-down strokes of a Teflon pestle homogenizer (clearance approx. 0.009 inch) using a Maxima Digital Overhead Stirrer (400 rpm; Fisher Scientific Co.). Homogenates were centrifuged (2000g for 10 minutes at 4°C), and the resulting supernatants were centrifuged (10,000g for 30 minutes at 4°C). Pellets were resuspended in 2 ml of 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4) and were subjected to osmotic shock by transfer of samples to tubes containing 7 ml of ice-cold MilliQ water. Samples were homogenized on ice with five up-and-down strokes of a Teflon pestle homogenizer. After 5 minutes, osmolarity was restored by transferring the samples to tubes containing 900 μl of 0.25 M HEPES and 900 μl of 1.0 M potassium tartrate dibasic hemihydrate solution. Samples were centrifuged (20,000g for 20 minutes at 4°C) and resulting supernatants centrifuged (55,000g for 1 hour at 4°C). To the resulting supernatants, 100 μl of 10 mM magnesium sulfate, 100 μl of 0.25 M HEPES, and 100 μl of 1.0 M potassium tartrate dibasic hemihydrate solution were added, followed by a final centrifugation (100,000g for 45 minutes at 4°C). Final pellets were resuspended in 2.4 ml of assay buffer (25 mM HEPES, 100 mM potassium tartrate dibasic hemihydrate, 50 μM EGTA, 100 μM EDTA, 1.7 mM ascorbic acid, and 2 mM ATP-Mg^{2+} , pH 7.4 adjusted dropwise with 10 M potassium hydroxide). Aliquots of the resulting suspension of isolated synaptic vesicles (100 μl) were added to tubes

containing assay buffer (300 μl), one of a range of concentrations of GZ-11608 (final concentration 0.1 nM to 0.1 mM; 50 μl) and 0.1 μM [^3H]DA (final concentration 10 nM; 50 μl) to obtain a final assay volume of 500 μl . After incubation for 8 minutes in a 37°C water bath (Reciprocal Shaking Bath Model 50; Precision Scientific, Chicago IL), [^3H]DA uptake was stopped by rapid filtration through presoaked (0.5% PEI for 1 hour at 4°C) Whatman GF/B Glass microfiber filters (1.0 μm pore size; Clifton, NJ) via a cell harvester (MP-43RS; Brandel Inc., Gaithersburg, MD). Subsequently, filters were washed three times with 4 ml of ice-cold wash buffer (25 mM HEPES, 100 mM potassium tartrate dibasic hemihydrate, 50 μM EGTA, 100 μM EDTA, 1.7 mM ascorbic acid, and 2 mM magnesium sulfate; pH 7.4 adjusted dropwise with 10 M potassium hydroxide). Scintillation cocktail (5 ml) was added to tubes containing the filters, followed by shaking for 30 minutes at room temperature. Radioactivity retained on the filters was determined by liquid scintillation spectrometry (TRI-CARB 2100 TR Packard scintillation counter; Packard BioScience Company, Meriden, CT).

Synaptosomal [^3H]DA and [^3H]5-HT Uptake. To evaluate the selectivity of GZ-11608 at VMAT2 relative to DAT and serotonin transporter (SERT), GZ-11608 inhibition of [^3H]DA and [^3H]5-HT uptake, respectively, into rat striatal synaptosomes was determined using previously published methods (Teng et al., 1997; Norrholm et al., 2007). Briefly, striata from individual rats were homogenized in 20 ml of 0.32 M sucrose containing 5 mM sodium bicarbonate (pH 7.4) with 16 up-and-down strokes of a Teflon pestle homogenizer (clearance approx. 0.003 inch) using the Maxima Digital Overhead Stirrer (400 rpm). Homogenates were centrifuged (2000g for 10 minutes at 4°C). Supernatants were centrifuged (20,000g for 17 minutes at 4°C) and pellets were resuspended (2.4 ml for DAT assay; 1.4 ml for SERT assay) in Krebs' buffer (125 mM sodium chloride, 5 mM potassium chloride, 1.5 mM magnesium sulfate, 1.25 mM calcium chloride, 1.5 mM monopotassium phosphate, 10 mM α -D-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline hydrochloride, and 0.1 mM ascorbic acid, and saturated with 95% O_2 /5% CO_2 ; pH 7.4 adjusted dropwise with 1 M sodium hydroxide). For DAT and SERT assays, aliquots of synaptosomal suspension (25 and 50 μl , respectively) were added to tubes containing Krebs' buffer (375 and 125 μl , respectively) and one of a range of concentrations of GZ-11608 (0, 0.1 nM to 0.1 mM) in 50 and 25 μl buffer, respectively. Uptake in the absence of GZ-11608 represents control. For nonspecific uptake, assay tubes contained nomifensine (final concentration, 100 μM in 50 μl for DAT assays) and fluoxetine (final concentration, 10 μM in 25 μl for SERT assays) in the absence of GZ-11608. For SERT assays, GBR-12935 (final concentration, 100 nM in 25 μl), a DAT inhibitor, was added to all assay tubes to prevent [^3H]5-HT uptake into dopaminergic terminals (Norrholm et al., 2007). DAT and SERT assay tubes (450 and 225 μl , respectively) were incubated at 34°C for 5 minutes. After incubation, tubes were placed on ice for 2 minutes. [^3H]DA (final concentration, 10 nM in 50 μl) or [^3H]5-HT (final concentration, 10 nM in 25 μl) was added to each tube. DAT and SERT assay tubes (final assay vol, 500 and 250 μl , respectively) were incubated at 34°C for 10 minutes. Uptake was stopped by addition of 3 ml of ice-cold assay buffer and subsequent filtration. [^3H]DA or [^3H]5-HT retained on the filters (presoaked in assay buffer containing 1 mM catechol for 1 hour at 4°C) was determined as previously described.

[^3H]Dofetilide Binding. GZ-11608 inhibition of [^3H]dofetilide binding to hERG assessed potential cardiotoxicity and selectivity of GZ-11608 for VMAT2 over hERG. HEK-293 cells stably expressing hERG channel protein were purchased from Millipore (Catalog number CYL3006; Billerica, MA). Binding assays were performed as previously described (Sviripa et al., 2014; Nickell et al., 2017). Briefly, frozen cells were thawed at 37°C and placed in T-75 cm^2 flasks (Becton Dickinson and Company, Franklin Lakes, NJ), containing 20 ml of complete media (minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 400 $\mu\text{g}/\text{ml}$ geneticin), according to the Millipore protocol. For 4–8 hours, cells adhered to flasks in a humidified atmosphere (5% CO_2 at 37°C), after

which the media was replaced with 20 ml fresh media. Subsequently, media was replaced every 48 hours. For routine passages, media was removed, cells rinsed with 2 ml of phosphate-buffered saline (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 2 mM potassium dihydrogen phosphate), followed by addition of Hank's balanced salt solution containing trypsin (0.5 g/l, porcine trypsin) and EDTA (0.5 mM). To dissociate the cells, flasks were placed in a 37°C incubator for 2–5 minutes, and then fresh complete media (5 ml) was added to the cell resuspensions, followed by seeding onto new flasks at $2\text{--}3 \times 10^6$ cells/flask. Passages were conducted every 6 days. At least three passages occurred before cell membrane collection. On the last passage prior to membrane preparation, cells were seeded onto 150- × 25-mm culture dishes at 2.5×10^6 cells/dish, and culture dishes were incubated (5% CO₂ at 37°C) for 40–48 hours. Media was removed and then culture dishes rinsed twice with 30°C Hanks' balanced salt solution (13 ml). A solution of ice-cold 0.32 M sucrose with 5 mM sodium bicarbonate (20 ml, pH 7.4) was then added to each culture dish on ice. Cells were scraped gently from the dishes and then homogenized (30 seconds) on ice with a Teflon pestle (approx. 0.003 inch) using a Maximal Digital homogenizer (280 rpm). Homogenates were centrifuged (300 and 800g for 4 minutes each at 4°C). Pellets were resuspended in 9 ml ice-cold MilliQ water, and osmolarity restored by addition of 1 ml of 500 mM Tris buffer (pH 7.4). Samples were centrifuged (20,000g for 30 minutes at 4°C). Pellets were resuspended in 2 ml assay buffer (50 mM Tris, 10 mM potassium chloride, and 1 mM magnesium chloride, pH 7.4, at 4°C). Aliquots of membrane suspension were stored at –80°C until use. To perform the [³H]dofetilide binding assay, membrane suspension was thawed and protein content determined using a Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA), with bovine albumin (Sigma-Aldrich Corporation) as the standard. Duplicate tubes were prepared containing membrane suspension (5 µg/100 µl), one of a range of concentrations of GZ-11608 (final concentrations 0, 0.1 nM to 0.1 mM in 25 µl) or amitriptyline (0, 0.1 nM–0.1 mM; positive control), assay buffer (150 µl), and [³H]dofetilide (5 nM in 25 µl) for a final assay volume of 250 µl. Amitriptyline (1 mM) was used to determine nonspecific binding (Teschemacher et al., 1999; Jo et al., 2000). Samples were incubated for 1 hour at room temperature. Reactions were stopped by rapid filtration through Whatman GF/B Glass microfiber filters presoaked in 0.5% PEI for 1 hour at 4°C. Filters were washed three times with 1 ml ice-cold assay buffer. Radioactivity retained by the filters was determined as previously described.

[³H]NIC and [³H]MLA Binding. To evaluate the selectivity of GZ-11608 for VMAT2 over $\alpha 4\beta 2$ and $\alpha 7$ nicotinic acetylcholine receptors (nAChRs), GZ-11608 inhibition of [³H]NIC and [³H]MLA binding was determined, respectively, using previously published methods (Horton et al., 2011a; Horton et al., 2011a). In brief, whole brains excluding cortex and cerebellum from individual rats were homogenized for 90 seconds in 20 vol of ice-cold assay buffer (2 mM HEPES, 14.4 mM sodium chloride, 0.15 mM potassium chloride, 0.2 mM calcium chloride, and 0.1 mM magnesium sulfate, pH 7.5 adjusted dropwise with 1 M sodium hydroxide) using a polytron. Homogenates were centrifuged (31,000g for 17 minutes at 4°C). Pellets were resuspended in 20 vol of assay buffer by sonication (Vibra Cell; Sonics & Materials Inc., Danbury, CT). Subsequently, duplicate samples were incubated in a 37°C water bath for 10 minutes, and then centrifuged (31,000g at 4°C for 17 minutes). Resulting pellets were resuspended in 20 vol of assay buffer by sonication, and centrifuged (31,000g for 17 minutes at 4°C). Final pellets were resuspended and stored in 10 ml of incubation buffer (20 mM HEPES, 144 mM sodium chloride, 1.5 mM potassium chloride, 2 mM calcium chloride, and 1 mM magnesium sulfate, pH 7.5 adjusted dropwise with 1 M sodium hydroxide) at –20°C until use. Thawed membrane suspensions (100–140 µg protein/100 µl) were added to tubes containing one of seven to nine concentrations of GZ-11608 (final concentration, 0, 0.1 nM to 0.1 mM in 50 µl) or nicotine (final concentration, 10 pM to 100 µM; positive control) or methyllycaconitine (final concentration, 10 pM to 100 µM; positive control), and [³H]NIC or [³H]MLA (final concentration, 3 nM in 50 µl), and incubation buffer (50 µl) for a final

assay volume of 250 µl. Nonspecific binding of [³H]NIC and [³H]MLA was determined in the presence of 10 µM of cytosine (50 µl) and 10 µM of nicotine (50 µl), respectively. Samples were incubated for 1 hour at room temperature. Unifilter-96 GF/B filter plates (1.0 µm pore size; PerkinElmer, Inc.) were presoaked in 0.5% PEI for 1 hour at 4°C. Reactions were stopped by filtration using a Packard Filter Mate Harvester (Perkin Elmer, Inc.). Plates were washed three times with 350 µl of ice-cold assay buffer, and dried for 1 hour at 45°C. Plates were bottom-sealed, and each well filled with 40 µl Microscint 20 cocktail. Bound radioactivity on the filter was determined via liquid scintillation spectrometry (Top Count NXT scintillation counter; PerkinElmer, Inc.).

Methamphetamine-Evoked [³H]DA Release. To further evaluate GZ-11608 efficacy as an inhibitor of the pharmacological effects of methamphetamine, the concentration-dependent effect of GZ-11608 to inhibit methamphetamine-evoked dopamine release from isolated striatal synaptic vesicles was determined using previously described methods (Teng et al., 1997; Horton et al., 2013). Also, the underlying mechanism of GZ-11608 inhibition was determined. Initially, the effect of GZ-11608 to release DA from synaptic vesicles was determined. Vesicles were prepared as described above for the vesicular DA uptake assay, except that final pellets were resuspended in a smaller volume (2.7 ml) of assay buffer. To preload the vesicles, [³H]DA (final concentration, 0.3 µM in 300 µl) was added to the vesicle suspension, and incubation proceeded at 37°C for 8 minutes. Samples were placed on ice for 2 minutes to stop [³H]DA uptake, and then centrifuged at 100,000g for 1 hour at 4°C to remove free [³H]DA not transported into the vesicles. Pellets were resuspended in a final volume of 4.2 ml of assay buffer. Aliquots of [³H]DA-preloaded vesicular suspension (180 µl) were added to tubes containing 1 of 11 concentrations of GZ-11608 (final concentrations, 0, 0.1 nM to 0.1 mM in 20 µl). Samples were incubated at 37°C for 8 minutes. Reactions were stopped by addition of 2.5 ml of ice-cold buffer (25 mM HEPES, 100 mM potassium tartrate, 50 µM EGTA, 100 µM EDTA, 1.7 mM ascorbic acid, 2 mM magnesium sulfate, pH 7.4), followed by rapid filtration onto PEI-pres soaked GF/B filters, and rinsing of the filters with ice-cold buffer (three times, 4 ml each). Scintillation cocktail was added, and radioactivity retained on the filters determined by liquid scintillation spectrometry. GZ-11608-evoked vesicular DA release was determined as the amount of [³H]DA retained by the vesicles subtracted from the amount retained in control vesicles not exposed to GZ-11608. To determine methamphetamine-evoked [³H]DA release and GZ-11608-induced inhibition of methamphetamine-evoked [³H]DA release, [³H]DA preloaded vesicles (180 µl) were incubated for 8 minutes at 37°C with 1 of 11 methamphetamine concentrations (final concentrations, 0, 0.1 µM to 20 mM in 10 µl) in the absence (control) and presence of a single concentration of GZ-11608 (final concentrations, 0, 10, 500 nM, and 10 µM in 10 µl) in a total volume of 200 µl. Reactions were stopped, radioactivity retained on the filter determined, and vesicular [³H]DA release calculated as described above to determine GZ-11608-induced inhibition of methamphetamine-evoked [³H]DA release.

Methamphetamine Sensitization. As a rapid means of determining if GZ-11608 decreases the in vivo effects of methamphetamine, locomotor sensitization following repeated methamphetamine injection was the initial assay employed (Alvers et al., 2012; Lee et al., 2018). Repeated methamphetamine administration results in a robust and stable increase in locomotor activity from day-to-day, allowing for reliable evaluation of the ability of compound to reduce the effects of methamphetamine on behavior. Locomotor activity was measured in a locomotor chamber (24 × 24 × 30 cm) with clear acrylic walls and floor. A horizontal 16 × 16 grid of photo beams was located 7 cm above the chamber floor, with each beam 2.5 cm apart. Movement in the chamber resulted in beam breaks, which were recorded and transformed into distance traveled (centimeter) by Versamax and Digipro System software (AccuScan Instruments Inc., Columbus, OH). The effect of GZ-11608 on methamphetamine-sensitized activity was determined using a mixed factor design with methamphetamine as

a between-subjects factor and GZ-11608 as within-subjects factor. Rats were assigned randomly to methamphetamine or saline groups. After a week of acclimation, rats were habituated to the apparatus by being placed in the chamber for 1 hour with no injection (day 0). On days 1–10, rats were injected (subcutaneous, s.c.) daily with either methamphetamine (1 mg/kg) or saline (1 ml/kg), immediately placed in the chamber, and activity measured for 1 hour. Methamphetamine dose and number of daily injections were chosen to provide stable, sensitized locomotor activity, on the basis of previous findings (Lee et al., 2018). On day 11, GZ-11608 (0, 1–30 mg/kg, s.c.) in a quasi-randomized dose order was injected 15 minutes prior to the daily methamphetamine or saline injection, and then, rats were placed immediately into the chamber for 1 hour. A washout period (2 to 3 days) intervened between testing of GZ-11608 doses to avoid potential drug accumulation. On washout days, methamphetamine or saline was injected and locomotor activity determined.

In a separate experiment employing a mixed factor design, the effect of GZ-11608 administration by oral gavage (p.o.) on methamphetamine locomotor sensitization was determined. Following repeated methamphetamine or saline for 5 days, rats were habituated to the oral gavage procedure. On days 5–10, rats received vehicle [15% (v/v) Kolliphor EL in saline, p.o., 3 ml] 15 minutes prior to methamphetamine or saline injection (subcutaneous) and were placed in the activity chamber for 1 hour (Wilmouth et al., 2013). On days 11–27, GZ-11608 (0, 17–300 mg/kg, p.o., ascending dose order) was administered, followed 15 minutes later by either methamphetamine or saline injection (1 ml/kg, s.c.), depending on group assignment, and immediate placement into the chamber for 1 hour. Between GZ-11608 doses, 2 to 3 days of washout occurred. On washout days, vehicle was administered by oral gavage and methamphetamine or saline was injected subcutaneously.

Striatal Dopamine Content. To determine whether GZ-11608 alters striatal dopamine content and/or exacerbates methamphetamine-induced striatal dopamine depletion, GZ-11608 was administered subcutaneously to rats in the absence and presence of a methamphetamine dose known to deplete rat striatal dopamine content (Bowyer et al., 1992, 1994; pilot study). The dose of GZ-11608 was chosen on the basis of its ability to reliably decrease methamphetamine-sensitized locomotor activity. Following acclimation to the colony and 3 days prior to drug injection, a thermal transponder (Bio Medic Data Systems, Inc., Seaford, DE) was implanted (subcutaneously) beneath the scapula to monitor body temperature. In the first series of experiments using a between-groups design, GZ-11608 (17 mg/kg, s.c.) or saline (1 ml/kg, s.c.) was administered 15 minutes prior to methamphetamine (30 mg/kg, i.p.) or saline (1 ml/kg, i.p.), according to random assignment to treatment group. In the second series of experiments also using a between-groups design, GZ-11608 or saline was administered 15 minutes after methamphetamine or saline. Methamphetamine was administered at 22°C ambient temperature in both series of experiments. Body temperature was monitored every 30 minutes for 8 hours following methamphetamine injection. If the body temperature increased to 41.3°C or higher, rats were transferred to a cage placed on ice until body temperature decreased to 40.0°C or below (Bowyer et al., 1992, 1994; Fukumura et al., 1998). Striata were obtained 72 hours after methamphetamine injection and were processed via high-performance liquid chromatography with electrochemical detection. Striata were weighed, placed in 1 ml of perchloric acid and sonicated. Tissue samples were centrifuged at 31,000g for 30 minutes at 4°C. Supernatant (50 μ l) was injected onto the octadecyl silica Ultrasphere C18 reverse-phase column (80 $\times$ 4.6 mm, 3 μ m; ESA Inc., Chelmsford, MA) via autosampler (508; Beckman Coulter, Inc., Fullerton, CA). Dopamine was detected by a coulometric-II detector with guard cell (model 5020; ESA, Inc.) maintained at +0.60 V and an analytical cell (model 5011) maintained at E1 = +0.05 V and E2 = +0.35 V. Mobile phase

(MP) consisted of 0.07 M citrate, 0.1 M acetate buffer with 175 mg/l octylsulfonic acid-sodium salt, 650 mg/l of sodium chloride, and 7% methanol (pH 4.2). Flow rate was 1.2 ml/min, and 4–5 minutes were required to analyze each sample. Dopamine standards were used to identify and quantify dopamine peak and amount using 32 Karat software (Beckman Coulter, Inc.).

Methamphetamine Self-Administration. The ability of GZ-11608 to dose dependently decrease the reinforcing effect of methamphetamine was determined using a within-subject design. Owing to the high doses of GZ-11608 required in the methamphetamine sensitization studies and the assumed low oral bioavailability, subcutaneous rather than oral administration was employed for self-administration studies. Two-lever operant chambers were used to train rats once daily for 3 days in 1-hour sessions to press a lever (active lever) for food pellet reinforcement (45 mg pellet, #F0021; BIO-SERV, Frenchtown, NJ) on a fixed ratio 1 (FR1) schedule, whereas responding on the other lever (inactive lever) had no programmed consequence. Subsequently, the operant schedule was incremented to an FR3 schedule for 3 days, then an FR5 schedule for 14 days until rats met the criteria for stable responding, which included: 1) ≥ 10 pellets earned/session and 2) a minimum of a 2:1 ratio of active/inactive lever presses. After delivery of each food reinforcer, the lights above both levers were illuminated for a 20-second signaled timeout period. After reaching stable responding for food on the FR5 schedule, rats underwent catheter implantation surgery. Rats were anesthetized (75 mg/kg ketamine, 7.5 mg/kg xylazine, and 0.75 mg/kg acepromazine; i.p.) and a silastic catheter was implanted into the jugular vein. The free end of the catheter was affixed with dental acrylic to the skull by metal screws and exited through the scalp. Rats were allowed to recover for 1 week. Prior to the start and at the end of each behavioral session. Catheters were flushed daily with 0.1 ml heparinized saline to maintain patency. Following recovery from surgery, rats were trained to press a lever for intravenous methamphetamine (0.05 mg/kg per infusion) during 1-hour daily sessions using a standard two-lever procedure as previously reported (Harrod et al., 2001; Beckmann et al., 2012). The FR schedule was incremented across training sessions (3 days, FR1; 3 days, FR3; 14 days, FR5). A 20-second signaled timeout occurred after each methamphetamine infusion. Upon reaching criteria for stable responding (≥ 10 infusions/session and a 2:1 ratio of active/inactive lever presses), a dose of GZ-11608 (0, 1, 3, 10, 17, and 30 mg/kg, in ascending dose order, s.c.) was administered 15 minutes prior to the session. GZ-11608 vehicle (0 mg/kg) was 15% Kolliphor in saline (1 ml/kg, s.c.).

Food-Maintained Responding. To evaluate the specificity of the GZ-11608-induced decrease in responding for intravenous methamphetamine, the ability of GZ-11608 to decrease food-maintained responding was determined using a within-subject design. GZ-11608 doses and pretreatment time were as described for methamphetamine self-administration experiments. Experiments were conducted as described above, with the exceptions that no surgery was performed and rats did not self-administer methamphetamine. Instead, rats were trained to a terminal FR5 to respond for food pellets (45 mg pellet, #F0021; BIO-SERV) in daily 1-hour sessions.

Repeated GZ-11608 Administration. A within-subject design was used to determine if tolerance developed to the GZ-11608-induced decrease in methamphetamine self-administration and/or food-maintained responding. One group of rats underwent operant training for food reinforcement, catheter implantation surgery, and operant training for intravenous methamphetamine (0.05 mg/kg per infusion) self-administration as described above. In these experiments, GZ-11608 (30 mg/kg, s.c.; a dose that reliably decreased methamphetamine self-administration) was administered 15 minutes prior to seven consecutive, daily methamphetamine self-administration sessions. Then, for five consecutive daily sessions, responding for methamphetamine was determined without GZ-11608 treatment. Another group of rats was trained for food-maintained responding,

and the effect of repeated GZ-11608 (30 mg/kg, s.c.) was determined using the same dose and pretreatment time as described above for methamphetamine self-administration.

Surmountability. To determine whether increasing the unit dose of methamphetamine would surmount the effect of GZ-11608 to decrease intravenous methamphetamine self-administration, another group of rats underwent operant training for food reinforcement, catheter implantation surgery, and operant training for methamphetamine (0.05 mg/kg per infusion) self-administration as described above. A within-subject design was employed to establish the methamphetamine dose-response across a range of methamphetamine doses (0.01–0.25 mg/kg per infusion) in the absence of GZ-11608. Each dose of methamphetamine was tested for three consecutive sessions. Then, the methamphetamine dose-response was re-evaluated in the same group of rats following treatment with GZ-11608 (30 mg/kg, s.c.) 15 minutes prior to the session. To maintain stable responding, two intervening maintenance sessions occurred between each session in which GZ-11608 was administered. For these maintenance sessions, no GZ-11608 treatment was administered prior to self-administration of each methamphetamine unit dose.

Reinstatement. The ability of GZ-11608 to decrease cue- and methamphetamine-induced reinstatement of methamphetamine-seeking behavior was determined using previously published methods (Harrod et al., 2003). In brief, three groups of rats were trained to self-administer methamphetamine (0.05 mg/kg per infusion) as described above, except that cue lights were illuminated for 5 seconds at the beginning of each session prior to presentation of the levers. For cue-induced reinstatement experiments, upon reaching the criteria for stable responding, rats underwent extinction for 14 days. During extinction, cue lights were not illuminated at the beginning or during daily 1-hour sessions, and active lever presses did not result in methamphetamine infusion. The day after the last extinction day (test for reinstatement), the cue light was illuminated at the beginning and during the session, and the dose effect for GZ-11608 to decrease cue-induced drug-seeking behavior was determined. Because drug-seeking behavior is diminished with repeated testing, two groups of rats were needed to generate the complete dose response. In one group, low doses (0, 3, 5.6, and 10 mg/kg, s.c.) were evaluated in a randomized order 15 minutes prior to the session, and higher doses (0, 10, and 17 mg/kg, s.c.) were evaluated in a second group. To maintain responding at extinction levels, five intervening sessions occurred between each session in which GZ-11608 was administered. On intervening sessions, there was no cue light illumination, no GZ-11608 pretreatment, and no methamphetamine infusion.

The effect of GZ-11608 (0, 10, 17, and 30 mg/kg, s.c.; in randomized order) on methamphetamine-induced reinstatement of drug-seeking behavior was determined in a third group of rats. GZ-11608 or saline was injected 15 minutes prior to the session, and methamphetamine (0.5 mg/kg, i.p.) was injected immediately prior to the session to reinstate drug-seeking behavior. Experiments were conducted using procedures similar to those in the experiments evaluating cue-induced reinstatement of drug seeking, except that the 20-second contingent cue light illumination continued during the 14 days of extinction, as well as on reinstatement tests.

Substitution of GZ-11608 for Methamphetamine. To determine whether GZ-11608 substitutes for methamphetamine in rats trained to self-administer intravenous methamphetamine, another experiment was conducted using a mixed factor design with GZ-11608 treatment as a between-subject factor, and dose and session as within-subject factors, similar to previously published methods (Harrod et al., 2003; Beckmann et al., 2012). Rats were trained to stable performance for methamphetamine (0.05 mg/kg per infusion) self-administration under an FR5 schedule of reinforcement as described above. Upon reaching the criteria for stable responding, rats were assigned randomly to either the GZ-11608 or saline groups. For the GZ-11608 group, responding on the active lever under the FR5 schedule resulted in intravenous infusions of GZ-11608 (0.01, 0.05, 0.1, and 0.5 mg/kg per infusion; in ascending order), each dose was administered across

Fig. 2. GZ-11608 potently and selectively inhibits VMAT2 relative to SERT, hERG, DAT, $\alpha 4\beta 2$ nAChRs, and $\alpha 7$ nAChRs. Chemical structure for GZ-11608 is shown in the insert. K_i values for GZ-11608 from the various neurochemical assays are provided in ascending order in the legend. Data are mean $\pm$ S.E.M. of specific uptake or binding as a percentage of the respective control (Con), in the absence of GZ-11608. Control values for each assay are as follows: VMAT2, 56.3 ± 10.9 pmol/min per milligram; SERT, 54.5 ± 7.16 fmol/min per milligram; hERG, 888 ± 80.3 fmol/mg; DAT, 91.0 ± 10.1 fmol/min per milligram; $\alpha 4\beta 2$ nAChRs, 21.5 ± 3.28 fmol/mg; $\alpha 7$ nAChRs, 31.4 ± 2.72 fmol/mg. $n = 4$ rats for neurotransmitter uptake assays, $n = 3$ cell batches for hERG binding assays, and $n = 3$ rats for nAChR binding assays.

four consecutive sessions. Only saline was available (intravenous) to the saline group across the same number of self-administration sessions. Then, for both GZ-11608 and saline groups, methamphetamine (0.05 mg/kg per infusion) was available for four consecutive self-administration sessions.

GZ-11608 Self-Administration. To determine whether GZ-11608 was self-administered in drug naive rats, a mixed factor design with GZ-11608 treatment as a between-subject factor, and dose and session as within-subject factors was conducted, similar to previously published methods (Harrod et al., 2003). Rats underwent operant training for food reinforcement, catheter implantation surgery, and random assignment to operant training of GZ-11608 or saline intravenous self-administration. For the saline group, only intravenous saline was available across the experiment. For the GZ-11608 group, each GZ-11608 dose (0.5, 0.1, and 0.05 mg/kg per infusion) was available in a descending order on an FR1 schedule of reinforcement for five consecutive days, followed by availability on an FR2 schedule for 3 days. To maintain stable responding, intervening maintenance sessions occurred after the evaluation of each GZ-11608 dose. During the maintenance sessions, rats responded for food reinforcement under FR1 for 2 days, and then under FR2 for 1 day; GZ-11608 was not available. As a positive control, ability to self-administer methamphetamine (0.05 mg/kg per infusion) under an FR1 for 5 days, FR2 for 3 days, and FR5 for 10 days was determined in the GZ-11608 group. To further evaluate GZ-11608 as a reinforcer, responding for intravenous GZ-11608 (0.1 mg/kg per infusion) or saline (depending on random group assignment) was determined in drug-naive rats under an FR1 for 5 days, FR2 for 3 days, and FR5 schedule for 10 days.

Pharmacokinetics. For dosing and pharmacokinetic sample collection, GZ-11608 was formulated in 15:85 Kolliphor EL/saline and was administered by oral gavage, intravenous bolus injection, or subcutaneous injection. Blood samples were collected in heparinized tubes from the saphenous vein and centrifuged at 4300g for 2 minutes.

Fig. 3. GZ-11608 evokes vesicular dopamine release (A) and competitively inhibits methamphetamine-evoked vesicular dopamine release (B). Data are mean $\pm$ S.E.M. [^3H] DA release from striatal vesicles as a percentage of control. Control (CON) values for [^3H]DA release in the absence of GZ-11608 or methamphetamine (METH) were 3290 ± 564 (A) and 3380 ± 708 dpm (B), determined in duplicate in each experiment. (A) EC_{50} and E_{max} for GZ-11608 are provided in the insert. (B) GZ-11608 concentrations are provided in the legend, and Schild regression and slope are shown in the insert. $n = 6$ rats, (A); $n = 4$ rats/assay, (B).

Plasma was collected and immediately frozen on dry ice followed by storage at -80°C until processing and analysis. An internal standard (ISTD) solution of 250 ng/ml of 1,4-diphenethylpiperidine (GZ-361B) in 50:50 methanol/Milli-Q water (v/v) was prepared from a stock solution of 100 $\mu\text{g}/\text{ml}$ in water. Independent stocks of GZ-11608 (100 $\mu\text{g}/\text{ml}$ in water) were prepared and used for calibrators and quality control samples. Ten spiking solutions [in 50:50 methanol/water (v/v)] were used for calibration curves ranging from 2.5 to 1000 ng/ml. Likewise, spiking solutions were used to prepare quality control samples at 7.5, 25, 500, and 850 ng/ml. Calibration and quality control samples consisted of 10 μl of spiking solution and 50 μl blank rat plasma. Experimental plasma samples were thawed on ice ($2-4^\circ\text{C}$), briefly vortexed, and diluted by the addition of 10 μl of sample and 10 μl of 50:50 methanol/water into 40 μl of blank rat plasma. Internal standard (15 μl of the 250 ng/ml ISTD solution) was added to samples and vortex mixed. All calibrators, quality control, and experimental samples were processed by protein precipitation with the addition of $2.4\times$ volume of methanol (180 μl) containing 0.1% formic acid, vortex mixed, stored at -20°C for 20 minutes, and centrifuged at $21,000g$ for 20 minutes at 4°C . Resulting supernatants were transferred into high-performance liquid chromatography vials for analyses.

For LC-MS analyses, samples were injected (10- μl volume) via an autosampler (15°C) and were analyzed for the positive-mode m/z 284.2/121.1 and 294.2/105.2 transitions of GZ-11608 and ISTD, respectively, on an AB Sciex Triple TOF (qTOF) 5600 unit (AB Sciex,

Framingham, MA) equipped with a Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD) controlled by Analyst TF software (ver. 1.7). Analyte and ISTD were resolved chromatographically (6 and 6.37 minutes, respectively). An Inertsil ODS-3 (4 μm , 3×100 mm) analytical column (GL Sciences, Rolling Hills Estates, CA) with a guard column of the same stationary phase was used. Analytes eluted using a gradient of aqueous 0.1% formic acid (MP-A) and acetonitrile (MP-B; gradient conditions: 30% MP-B to 75% MP-B linear ramp over 3 minutes, then held at 75% MP-B for 1 minute, followed by 3 minutes linear ramp back to 30% MP-B and equilibration at 30% MP-B for 5 minutes) at a 0.2 ml/min total flow rate. Eluent was routed through the qTOF TurboIonSpray ESI probe thru an integrated Valco diverter valve (VICI Valco Instruments, Huston, TX) between 5 and 7.3 minutes and vaporized/ionized using a source temperature of 550°C and an ion spray voltage of 4500 V (gas settings at GS1 = 35/GS2 = 35/CUR = 30). qTOF method consisted of two repeat experiments scanning the 100–300 amu mass range for product ions of GZ-11608 (m/z 284.15; DP = 80/CE = 35/CES = 0/IRD = 67/IRW = 25) or ISTD (m/z 294.2; DP = 70/CE = 35/CES = 0/IRD = 67/IRW = 25). Quantitation was conducted using MultiQuant software (ver. 3.0; AB Sciex) on the extracted 121.1 and 105.2 m/z product ion peak areas, with calibration curves constructed from the analyte to ISTD peak area and concentration ratios (best fit line from linear regression weighted at $1/Y^2$). Concentrations of GZ-11608 in experimental samples were interpolated from the corresponding curve;

Fig. 4. Locomotor sensitization following repeated methamphetamine administration (A) is decreased in a dose-dependent manner by GZ-11608 [subcutaneous, (B); oral, (C)]. Data are mean $\pm$ S.E.M. of distance traveled in meters (m) during the last 45 minutes of the 60-minute sessions. (A) Day 0 shows activity on a habituation day prior to the first methamphetamine or saline injection. Methamphetamine (1 mg/kg, s.c.) or saline (1 ml/kg, s.c.) was administered once daily for 10 days (days 1–10). Locomotor activity for the saline group is shown in the insert. (B and C) GZ-11608 or vehicle [Veh, 15% (v/v) Kolliphor EL/saline; 1 ml/kg] was administered subcutaneously (B) or by oral gavage (C) 15 minutes prior to methamphetamine (METH; 1.0 mg/kg, s.c.) or saline injection. Dashed line

otherwise, samples outside the calibration range were reprocessed at 1:4 or 1:10 dilution with blank rat plasma alongside likewise diluted and processed quality control. Samples were analyzed following injection of a calibration curve and quality control samples. Three of four quality control samples were required to be within 15% of nominal values. Quality controls were injected every 24 experimental samples and at the end of the sample sequence. The lower limit of quantitation was 2.5 ng/ml and the upper limit of quantitation was 1000 ng/ml. Accuracy and precision were within 15%.

Brain Tissue Collection and Processing for HPLC with Tandem Mass Spectrometry Analysis. Following administration of GZ-11608 (30 mg/kg, s.c.), rats were placed under isoflurane (USP; Henry Schein, Dublin, OH) anesthesia and prepared for perfusion with 1:99 (v/v) heparin in 0.9% saline as previously described (Gage et al., 2012). Brains were collected at 5, 30, and 45 minutes, and at 1, 3, 6, 8, and 12 hours after dosing. To remove systemic blood, rats were perfused at 20 ml/min until the liver was cleared of blood. The brain was removed with the aid of rongeurs and homogenized 1:2 (w/v) with ice-cold phosphate-buffered saline (137 mM sodium chloride, 2.7 mM potassium chloride, 1.0 mM sodium hydrogen phosphate, and 1.8 mM potassium dihydrogen phosphate). Homogenates were stored at -80°C until analysis. Calibration, quality control, and experimental brain samples were thawed and vortex mixed. A 50- μl aliquot was added to 15 μl of internal standard (250 ng/ml GZ-361B in methanol/water 50:50). Samples were treated with 200 μl of 0.1% formic acid in methanol to precipitate proteins. Samples were vortex mixed (10 seconds), chilled (-20°C for 20 minutes), remixed (10 seconds), and centrifuged (13,000g for 18 minutes at 4°C). Resulting supernatants were collected into amber HPLC vials fitted with 150- μl glass inserts, and immediately analyzed for analyte content by LC with tandem mass spectrometry as described for the plasma analyses.

Data Analysis. Specific [^3H]DA uptake, [^3H]5-HT uptake, [^3H]dofetilide binding, [^3H]NIC binding, and [^3H]MLA binding were calculated by subtracting nonspecific uptake or binding from total uptake or binding, respectively. The GZ-11608 concentration that produced 50% inhibition of specific uptake or binding (IC_{50} values) was obtained from individual concentration-response curves via an iterative curve-fitting program (Prism 7.03; GraphPad Software, Inc., La Jolla, CA). Inhibition constants (K_i values) were determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The selectivity ratio for GZ-11608 relative to the off-target sites was determined as the K_i value for inhibition of VMAT2 divided by the K_i value for DAT, SERT, hERG, and nAChRs, respectively. EC_{50} values from individual concentration-response curves for methamphetamine or GZ-11608 to evoke [^3H]DA release from synaptic vesicles was determined using Prism 7.0. The mechanism of GZ-11608-induced inhibition of methamphetamine-evoked vesicular [^3H]DA release was determined using Schild analysis. Dose ratios (DR) were obtained by dividing the EC_{50} for methamphetamine-evoked [^3H]DA release in the presence of each concentration of GZ-11608 by that in the absence of GZ-11608. Log (DR-1), plotted as a function of log GZ-11608 concentration, provided the Schild regression; linearity of the slope was significantly different from unity if the 95% confidence intervals (CI) did not include 1.0 (Prism 7.03; Kenakin et al., 2006).

represents 50% of the distance traveled following vehicle. Locomotor activity for the saline group following GZ-11608 or vehicle is shown in (B) and (C) inserts. $^*P < 0.05$ compared with day 0; $^{\#}P < 0.05$ compared with day 1 within group (A); $^*P < 0.05$ compared with vehicle within groups (B) and (C); two-way mixed-factor ANOVA followed by Tukey's post-hoc test; $n = 10$ rats/group in (A), which were subdivided into $n = 5$ rats/group for (B) and (C). Note: Methamphetamine was administered inadvertently to two of the rats in the saline group and saline was administered inadvertently to one rat in the methamphetamine group on the day before the 300 mg/kg dose of GZ-11608 (C), and these data were not included in the analysis.

For all behavioral experiments, distance traveled and number of responses were subjected to repeated measures analysis of variance (ANOVA), followed by Tukey's post-hoc tests when appropriate, unless otherwise indicated.

Pharmacokinetic data were evaluated graphically using GraphPad (Prism 8.0) and analyzed using Phoenix 64 (Certara USA, Inc., Princeton, NJ) to estimate pharmacokinetic parameters. Noncompartmental methods were used to estimate C_{max} , time of maximum concentration (T_{max}), area under the curve ($AUC_{0 \rightarrow \infty}$), elimination half-life ($t_{1/2}$), and clearance or clearance/F, where F is the bioavailability. Bioavailability estimates of the oral and subcutaneous routes were determined using the respective AUCs and doses in comparison with the intravenous bolus data using the following equation:

$$\%F = \frac{AUC_{e.v.} \cdot Dose_{i.v.}}{AUC_{i.v.} \cdot Dose_{e.v.}} \cdot 100 \quad (1)$$

Where i.v. and e.v. denote intravenous and extravascular routes. Linear regression was performed to estimate whether the slope of dose-versus-PK parameters deviated from zero (GraphPad).

Results

GZ-11608 Potently and Selectively Inhibits VMAT2 Function Relative to Interactions at Off-Target Sites. GZ-11608 potently inhibited ($K_i = 25 \pm 4$ nM) [3H]DA uptake at VMAT2 with maximal inhibition (I_{max}) of >95% (Fig. 2). GZ-11608 selectively inhibited [3H]DA uptake at VMAT2, having at least 92-fold higher affinity at VMAT2 relative to off-target sites (Fig. 2; [3H]5-HT uptake at SERT: $K_i = 2.36 \pm 0.29$ μ M, $I_{max} > 95\%$, 92-fold selective for VMAT2; [3H]dofetilide binding to hERG: $K_i = 4.16 \pm 1.68$ μ M, $I_{max} > 90\%$, 163-fold selective for VMAT2; and, [3H]DA uptake at DAT: $K_i = 6.15 \pm 0.74$ μ M, $I_{max} > 90\%$, 241-fold selective for VMAT2). GZ-11608 did not inhibit [3H]NIC and [3H]MLA binding to $\alpha 4\beta 2$ and $\alpha 7$ nAChRs, respectively ($K_i > 30$ μ M; >1180-fold selective for VMAT2). Taken together, GZ-11608 exhibited high affinity and selectivity for VMAT2 over SERT, hERG, DAT, $\alpha 4\beta 2$ nAChRs, and $\alpha 7$ nAChRs.

GZ-11608 Evokes Vesicular [3H]DA Release and Inhibits Methamphetamine-Evoked Vesicular [3H]DA Release. Effects of GZ-11608 to release vesicular [3H]DA and to inhibit methamphetamine-evoked vesicular dopamine release are illustrated in Fig. 3. GZ-11608 (0.1 nM to 0.1 mM) stimulated [3H]DA release from isolated striatal synaptic vesicles with an EC_{50} value of 0.62 ± 0.14 μ M and an E_{max} value of $76.8\% \pm 5.0\%$. The GZ-11608 concentration-response data fit a one-site model (nonlinear regression, $R^2 = 0.87$, $P < 0.05$; Fig. 3A). The effect of GZ-11608 to inhibit methamphetamine-evoked [3H]DA release in the absence and presence of low, medium, and high concentration of GZ-11608 is illustrated in Fig. 3B. Methamphetamine evoked [3H]DA release from isolated vesicles with an EC_{50} value of 14.5 ± 4.10 μ M, an E_{max} value of $90.0\% \pm 2.04\%$, and in the absence of GZ-11608, the methamphetamine concentration-response data fit a one-site model (nonlinear regression, $R^2 = 0.97$, $P < 0.0001$; Fig. 3B). Kinetic parameters for methamphetamine-evoked [3H]DA release from striatal synaptic vesicles were consistent with previously published values (Horton et al., 2013). Concentration-response data for methamphetamine in the presence of varying GZ-11608 concentrations (10, 500 nM, or 10 μ M) each fit a one-site model

Fig. 5. GZ-11608 does not exacerbate methamphetamine-induced striatal dopamine depletion. Data are mean $\pm$ S.E.M. striatal DA content expressed as nanograms per milligram tissue. GZ-11608 (17 mg/kg, s.c.) was administered (A) 15 minutes prior to methamphetamine (METH, 30 mg/kg, i.p.) or saline (1 ml/kg, i.p.; $n = 7$ –12 rats/group) or (B) 15 minutes after METH (30 mg/kg, i.p.) or saline (1 ml/kg, i.p.; $n = 4$ –8 rats/group). As a result of the lethality associated with methamphetamine, data were not collected for 18 of the 80 rats in the experiment (five in the METH/saline group; five in the METH/GZ-11608 group; three in the saline/METH group; and four in the GZ-11608/METH group). * $P < 0.05$ compared with the respective saline/saline control group; one-way between-groups ANOVA followed by Tukey's post-hoc test.

(nonlinear regression, $R^2 = 0.94$, 0.95 , 0.90 , respectively, multiple P values < 0.0001 ; Fig. 3B). Methamphetamine concentration-response curves for vesicular [3H]DA release were shifted rightward, and EC_{50} values in the presence of GZ-11608 (0, 10, 500 nM, and 10 μ M) were 14.5 ± 4.10 , 17.3 ± 4.15 , 122 ± 32.3 , and 388 ± 23.7 μ M, respectively; whereas, the E_{max} values were not altered as the concentration of GZ-11608 increased ($90.0\% \pm 2.04\%$, $94.1\% \pm 1.25\%$, $88.1\% \pm 1.27\%$, and $88.4\% \pm 3.70\%$, respectively). Two-way ANOVA revealed a methamphetamine and GZ-11608 interaction [$F_{33,99} = 11.2$, $P < 0.0001$]. The Schild regression had a slope of 0.90 (CI: 0.603 to 1.20; Fig. 3B insert), consistent with a competitive mechanism of VMAT2 inhibition.

GZ-11608 Decreases Methamphetamine Sensitization. GZ-11608 decreased methamphetamine sensitization (1.0 mg/kg, s.c., once daily for 10 days; Fig. 4). Locomotor activity

Fig. 6. GZ-11608 specifically decreases responding for intravenous methamphetamine, without altering responding for food. GZ-11608 or vehicle [Veh, 15% (v/v) Kolliphor EL in saline, 1 ml/kg] was administered (subcutaneously) to rats trained to (A) self-administer methamphetamine (METH, 0.05 mg/kg per infusion) or (B) to respond for food pellet reinforcers during 60-minute FR5 operant sessions. Data are mean $\pm$ S.E.M. number of reinforcers earned as a percentage of the respective vehicle control [Veh; 16.4 $\pm$ 2.6 methamphetamine infusions, (A); 38.4 $\pm$ 5.0 food pellets, (B)]. Dotted line represents 50% of the reinforcers earned following vehicle injection. The complete GZ-11608 dose-response curve was not collected for one rat in the methamphetamine self-administration experiment owing to an insecure head-mount; data following head mount loss were not included in the analysis. * P < 0.05 compared with vehicle control; n = 8–9 rats, (A); n = 11 rats, (B); one-way repeated-measures ANOVAs followed by Tukey's post-hoc test.

(distance traveled) in the methamphetamine-treated group increased following acute methamphetamine and plateaued after five daily methamphetamine injections at an activity level greater than that observed following acute injection on day 1 (i.e., sensitization; Fig. 4A). Across the same time period, locomotor activity in the saline-injected control group was not changed (Fig. 4A, inset). Two-way ANOVA revealed a treatment $\times$ session interaction [$F_{10,90} = 21.7$, P < 0.001]. Post-hoc analysis revealed no differences between the treatment groups on the initial habituation day (day 0). However, as expected, methamphetamine increased distance traveled on day 1 relative to the saline-injected group (P < 0.001) and distance traveled was greater on days 5–10 than on day 1 in the methamphetamine treatment group (p s < 0.05), but not in the saline treatment group. There were no significant differences in activity between days 5 and 10 in the methamphetamine treatment group, indicating that sensitization occurred and stabilized by day 5.

Fig. 7. Repeated GZ-11608 decreases intravenous methamphetamine self-administration, without the development of tolerance. Baseline represents the number of methamphetamine infusions (22.2 $\pm$ 4.0) after vehicle injection [15% (v/v) Kolliphor EL in saline, 1 ml/kg] 15 minutes prior to 60-minute FR5 sessions. GZ-11608 (30 mg/kg, s.c., once daily for 7 days) was administered 15 minutes prior to methamphetamine (METH) self-administration sessions, followed by five methamphetamine self-administration sessions with no GZ-11608 treatment. Data are presented as mean $\pm$ S.E.M. of methamphetamine infusions earned as a percentage of baseline. Dotted line represents 50% of baseline responding for methamphetamine and dashed line represents 100% of baseline. GZ-11608 treatment decreased (55%–75%) responding for methamphetamine. Responding for methamphetamine returned to baseline levels after discontinuation of GZ-11608 treatment. Complete data were not collected for two rats owing to insecure head mounts; data following head mount loss were not included in the analysis. * P < 0.05 compared with baseline; one-way repeated-measures ANOVA followed by Tukey's post-hoc test; n = 5–7 rats.

GZ-11608 (subcutaneous) decreased methamphetamine-sensitized locomotor activity in a dose-dependent manner without altering activity in the saline-injected control group (Fig. 4B). Two-way ANOVA revealed an interaction between methamphetamine treatment and GZ-11608 dose [$F_{5,28} = 23.5$, P < 0.001]. Post-hoc analysis revealed that GZ-11608 (10, 17, and 30 mg/kg) decreased methamphetamine sensitization relative to vehicle (15% Kolliphore EL in saline) (P < 0.005), and activity reached the criterion of a 50% reduction relative to activity following vehicle (Fig. 4B). In the saline-injected control group, no effect of GZ-11608 at any dose relative to vehicle was found (Fig. 4B, inset), indicating a specific effect of GZ-11608 on the methamphetamine-sensitized response. Activity at the highest dose of GZ-11608 (30 mg/kg) in the methamphetamine group was not different from activity following vehicle injection in the saline group, demonstrating GZ-11608 blockade of the expression of methamphetamine sensitization.

The effect of GZ-11608 following oral administration on methamphetamine sensitization was determined. GZ-11608 (17–300 mg/kg, p.o.) decreased methamphetamine sensitization in a dose-dependent manner relative to vehicle; however, activity tended to increase following oral administration of GZ-11608 in the saline-injected control group (Fig. 4C). An interaction was found between methamphetamine treatment and GZ-11608 dose [$F_{6,53} = 10.1$, P < 0.001] (Fig. 4C). Post-hoc analysis revealed that GZ-11608 (300 mg/kg) decreased sensitized locomotor activity relative to vehicle in the methamphetamine treatment group (P < 0.05; Fig. 4C). Activity following the highest dose (300 mg/kg) of GZ-11608 reached the criterion of a 50% reduction relative to vehicle. Activity

Fig. 8. Increasing the unit dose of self-administered intravenous methamphetamine did not surmount the GZ-11608-induced decrease in responding for methamphetamine. Data are presented as mean $\pm$ S.E.M. of methamphetamine (METH) or saline infusions earned during 60-minute FR5 operant sessions. The initial training dose of methamphetamine was 0.05 mg/kg per infusion, followed by varying unit doses of methamphetamine or saline presented in a randomized order of presentation. Open circles represent no GZ-11608 treatment, and closed circles represent GZ-11608 (30 mg/kg, s.c.) treatment 15 minutes prior to the session. To maintain stable responding, two intervening maintenance sessions occurred between each GZ-11608 treatment session, in which methamphetamine was available and no GZ-11608 was administered. Complete data were not collected for one rat owing to an insecure head-mount; data following head mount loss were not included in the analysis. * $P < 0.05$ compared with saline infusion for the respective group; * $P < 0.05$ compared with the no treatment condition for each unit dose of methamphetamine; two-way mixed-factor ANOVA followed by Tukey's post-hoc test; $n = 6$ to 7 rats.

following GZ-11608 (17–300 mg/kg) in the saline-injected group tended to increase but did not reach statistical significance (Fig. 4C; inset). Activity at the highest dose of GZ-11608 (300 mg/kg) in the methamphetamine group was not different from activity following vehicle in the saline group, demonstrating GZ-11608 blockade of the expression of methamphetamine sensitization. Taken together, GZ-11608 following subcutaneous and oral gavage administration specifically decreased methamphetamine sensitization.

GZ-11608 Does Not Alter Striatal Dopamine Content and Does Not Exacerbate the Decrease in Dopamine Content Produced by Methamphetamine. To determine the effect of GZ-11608 on striatal dopamine content, a dose of GZ-11608 (17 mg/kg, s.c.) that both reliably and specifically decreases methamphetamine sensitization was employed (Fig. 5). One-way ANOVAs revealed significant differences in dopamine content after either GZ-11608 pretreatment or post-treatment and a relatively high dose (30 mg/kg, i.p.) of methamphetamine (pretreatment, $F_{3,34} = 6.26$, $P < 0.05$, Fig. 5A; post-treatment, $F_{3,21} = 16.5$, $P < 0.001$, Fig. 5B). Post-hoc analysis revealed that dopamine content was decreased (40%–50%) following methamphetamine alone compared with the respective saline control groups. GZ-11608 alone did not alter dopamine content compared with the respective saline control. Dopamine content following GZ-11608 pretreatment or post-treatment and methamphetamine was not different from that following methamphetamine alone (Fig. 5, respectively), indicating that GZ-11608 did not exacerbate the effect of methamphetamine to decrease striatal dopamine content.

Fig. 9. GZ-11608 dose dependently decreases cue-induced and methamphetamine-induced reinstatement of methamphetamine seeking. Data are presented as mean $\pm$ S.E.M. of number of lever presses. Baseline (BL) represents lever presses on the last day of maintenance, during which methamphetamine (0.05 mg/kg per infusion) was available for self-administration. For cue-induced reinstatement (A), extinction (Ext) represents lever presses on the last 14 days in which no cue light was presented and no methamphetamine was available. GZ-11608 treatment decreased cue-induced lever presses relative to vehicle injection (0 dose; Kolliphor EL). Number of cue-induced lever presses following GZ-11608 (10 mg/kg) was not different between the two groups of rats evaluated ($P > 0.05$), and these data were combined for analysis. Between each session in which the effect of GZ-11608 was evaluated, five extinction sessions occurred. For methamphetamine-induced reinstatement (B), extinction (Ext) represents lever presses on the last day of 14 days in which the cue light was presented, but no methamphetamine was available. GZ-11608 decreased methamphetamine-induced lever presses relative to vehicle injection (0 dose). Between each session in which the effect of GZ-11608 was evaluated, five extinction sessions occurred. No reinstatement was exhibited by three rats (defined as <10 cue-induced responses following vehicle); data from these rats were not included in the analysis. The complete GZ-11608 dose-response curve was not collected for two rats in the cue-induced reinstatement experiment (A) owing to an insecure head-mount; data following head mount loss were not included in the analysis. * $P < 0.05$ compared with the respective vehicle control, # $P < 0.05$ compared with respective last day of extinction prior to GZ-11608 treatment; one-way repeated-measures ANOVAs followed by Tukey's post-hoc test; $n = 6$ –12 rats/experiment.

GZ-11608 Decreases Responding for Intravenous Methamphetamine, but Not for Food. To determine if GZ-11608 specifically decreases methamphetamine self-administration, the effects of GZ-11608 (1–30 mg/kg, s.c.) on methamphetamine (0.05 mg/kg per infusion) self-administration and food-maintained responding were evaluated. One-way ANOVAs revealed that GZ-11608 dose-dependently decreased methamphetamine self-administration but not food-maintained responding [$F_{5,45} = 3.92$, $P < 0.005$, Fig. 6A; ($F_{5,60} = 3.23$, $P = 0.0119$), Fig. 6B, respectively]. Post-hoc analysis revealed that after the highest dose of GZ-11608 (30 mg/kg), responding for methamphetamine was lower than following vehicle ($P < 0.05$, Fig. 6A). Post-hoc analysis also revealed that GZ-11608 did not alter responding for

Fig. 10. GZ-11608 does not substitute for methamphetamine self-administration. Data are presented as mean $\pm$ S.E.M. number of intravenous infusions [methamphetamine (METH), GZ-11608, or saline] across 60-minute FR5 sessions. Baseline represents the number of methamphetamine infusions on the last day of maintenance for both the GZ-11608 and saline groups. Ascending doses of GZ-11608 for the GZ-11608 group or saline for the saline group were available as reinforcers for four sessions/unit dose. Then, methamphetamine was available during the final four sessions of the experiment. $n = 4$ rats/group.

food ($P > 0.05$, Fig. 6B). Thus, GZ-11608 specifically decreased methamphetamine self-administration.

Tolerance Does Not Develop to the Effect of GZ-11608 to Decrease Responding for Intravenous Methamphetamine. One-way ANOVA revealed that following acute administration GZ-11608 (30 mg/kg, s.c.) decreased responding for methamphetamine (0.05 mg/kg per infusion) and that responding continued to be decreased across seven consecutive, once-daily GZ-11608 treatments ($F_{7,43} = 3.68$, $P < 0.05$), indicating that tolerance did not develop to this effect of GZ-11608 (Fig. 7). Upon cessation of GZ-11608 treatment, responding for methamphetamine returned to baseline ($F_{5,26} = 3.67$, $P < 0.05$; Fig. 7). Post-hoc analysis revealed that responding during post-treatment sessions 1 and 2 was decreased compared with baseline ($P < 0.05$), and that responding during post-treatment sessions 3–5 was not different from baseline ($P > 0.05$). Of note, repeated administration of GZ-11608 (30 mg/kg, s.c., once daily for 7 days) initially decreased responding for food but tolerance developed to this effect after five daily administrations (Supplemental Fig. 1).

Increasing the Unit Dose of Methamphetamine Does Not Surmount the Effect of GZ-11608 to Decrease Responding for Intravenous Methamphetamine. This experiment determined if the GZ-11608-induced decrease in methamphetamine self-administration could be surmounted by increasing the unit dose of methamphetamine. Inverted U-shaped methamphetamine dose-response curves were obtained with no treatment as well as following GZ-11608 (30 mg/kg, s.c.) treatment. Following GZ-11608 treatment, the methamphetamine dose-response curve was shifted downward and rightward relative to the curve obtained with no treatment (Fig. 8). Two-way ANOVA of the data expressed as number of methamphetamine infusions revealed an interaction between GZ-11608 treatment and methamphetamine unit dose [$F_{5,68} = 2.55$, $P < 0.05$]. Post-hoc analysis revealed that GZ-11608 decreased the number of methamphetamine infusions when low unit doses (0.01–0.05 mg/kg per infusion) were available, whereas GZ-11608 had no effect on number of infusions when higher unit doses (0.1 and 0.25 mg/kg per infusion) of methamphetamine were available. Without GZ-11608 treatment, the peak number of methamphetamine infusions (20 infusions) occurred with a unit dose of 0.025 mg/kg per infusion. Following GZ-11608 treatment,

the peak number (nine) of methamphetamine infusions occurred with 0.1 mg/kg per infusion, representing an approx. 50% decrease relative to no GZ-11608 treatment (Fig. 8). Thus, increasing the methamphetamine unit dose did not surmount the effect of GZ-11608.

GZ-11608 Decreases Cue- and Methamphetamine-Induced Reinstatement of Methamphetamine-Seeking Behavior. These experiments determined if GZ-11608 decreases cue- and methamphetamine-induced reinstatement (models of relapse). In a dose-related manner, GZ-11608 decreased cue- and methamphetamine-induced reinstatement of methamphetamine seeking (Fig. 9). For both cue- and methamphetamine-induced reinstatement experiments, one-way ANOVAs revealed that methamphetamine seeking was decreased in a dose-related manner by GZ-11608 [$F_{6,99} = 34.5$, $P < 0.001$] and ($F_{5,54} = 17.1$, $P < 0.001$), respectively]. Post-hoc analyses revealed that lever pressing was decreased following 14 extinction sessions compared with baseline and that both the cue and methamphetamine (0.5 mg/kg, i.p.) reinstated methamphetamine seeking following vehicle pretreatment (multiple P values < 0.05). GZ-11608 dose dependently decreased both cue- and methamphetamine-induced reinstatement ($ps < 0.001$). Thus, GZ-11608 dose dependently decreased cue- and methamphetamine-induced reinstatement of methamphetamine seeking.

GZ-11608 Does Not Substitute for Methamphetamine. One potential mechanism underlying the GZ-11608-induced decrease in methamphetamine self-administration may be an action as a substitute reinforcer. In rats trained to self-administer methamphetamine, methamphetamine was replaced by either intravenous GZ-11608 or saline. Figure 10 illustrates that responding across a range of GZ-11608 doses was not different from responding for saline. Two-way ANOVA revealed no main effect of treatment ($F_{1,3} = 0.206$, $P = 0.681$) and no treatment and session interaction ($F_{20,60} = 0.368$, $P = 0.99$); however, there was a main effect of session ($F_{20,60} = 7.46$, $P < 0.001$). Post-hoc analysis revealed no differences between the groups at baseline ($P > 0.05$) and no differences between groups responding for the range of GZ-11608 doses and saline across sessions 1–16. During sessions 17–20, in which methamphetamine was available and responding returned to baseline, there were also no between-groups differences

Fig. 11. GZ-11608 is not self-administered by drug-naïve rats. Responding during successive operant sessions is illustrated across (A–D), with the exception of three intervening sessions between each GZ-11608 unit dose and prior to the methamphetamine self-administration sessions, in which responding for food pellet reinforcers occurred (data not shown). Data are presented as mean $\pm$ S.E.M. number of intravenous infusions of GZ-11608 (descending dose order) for the GZ-11608 group or saline (1 ml/kg per infusion, i.v.) for the saline group across 60-minute FR1 and FR2 sessions (A–C). Methamphetamine self-administration was available to the GZ-11608 group, and saline was available to the saline group under the FR1, FR2, and FR5 schedules of reinforcement (D). Complete data were not

in responding. Thus, GZ-11608 did not substitute for methamphetamine as a reinforcer.

GZ-11608 Does Not Serve as a Reinforcer. To assess the abuse liability of GZ-11608, drug naïve rats were trained to respond for GZ-11608 using the same procedure as that used for methamphetamine self-administration. Figure 11 shows that GZ-11608 was not self-administered and may have had aversive properties at the highest dose evaluated. Two-way ANOVA on the number of responses at the highest GZ-11608 dose (0.5 mg/kg per infusion) revealed a main effect of session ($F_{7,70} = 4.50$, $P < 0.05$), a trend for a GZ-11608 and session interaction ($F_{7,70} = 2.01$, $P = 0.0658$), but no main effect of GZ-11608 ($F_{1,10} = 2.30$, $P = 0.160$). Post-hoc analysis revealed that responding during sessions 1 and 2 was decreased when GZ-11608 (0.5 mg/kg per infusion) was available compared with the saline group ($P < 0.05$), supporting a conclusions that the high dose of GZ-11608 served as a punisher (Fig. 11A). Analysis of the intermediate dose of GZ-11608 (0.1 mg/kg per infusion) revealed no main effects of treatment or session ($F_{1,10} = 0.51$, $P = 0.492$ and $F_{7,70} = 2.09$, $P = 0.056$, respectively), and no treatment and session interaction ($F_{7,70} = 0.42$, $P = 0.884$; Fig. 11B). Analysis of the lowest dose of GZ-11608 (0.05 mg/kg per infusion) revealed no main effects of treatment or session ($F_{1,9} = 0.69$, $P = 0.426$; $F_{7,59} = 1.92$, $P = 0.082$, respectively) and no treatment and session interaction ($F_{7,59} = 0.20$, $P = 0.984$; Fig. 11C). Thus, responding for GZ-11608 (0.05–0.1 mg/kg per infusion) was not different from the saline group. The lack of responding for GZ-11608 was not attributable to lack of intravenous catheter patency, because, as expected, responding increased subsequently when methamphetamine was available (Fig. 11D). During the FR1/FR2 components of the session, a main effect of session ($F_{7,42} = 2.56$, $P < 0.05$) was found, with no main effect of treatment ($F_{1,6} = 2.87$, $P = 0.14$) and no treatment and session interaction ($F_{7,42} = 1.22$, $P = 0.31$). During the FR5 component, a main effect of treatment ($F_{17,102} = 2.09$, $P < 0.05$) was found with no main effect of session ($F_{1,6} = 5.66$, $P = 0.055$) and no treatment and session interaction ($F_{17,102} = 1.11$, $P = 0.37$; Fig. 11D). Thus, GZ-11608 does not have reinforcing properties.

Pharmacokinetics. To assess oral bioavailability, GZ-11608 was administered by gavage (40 mg/kg, p.o.) and by tail vein bolus injection (5 mg/kg, i.v.). As shown in Fig. 12A, following oral dosing, GZ-11608 reached maximum concentrations within 2 hours, and its disposition, after oral and intravenous doses, followed monoexponential decay. Oral bioavailability of GZ-11608 was 3.6% (Table 1). Likewise, after subcutaneous dosing (30 mg/kg), GZ-11608 followed monoexponential decay, but maximum concentrations were within 2–4 hours after administration (Fig. 12B). To assess the pharmacokinetics over doses that provided behavioral efficacy, GZ-11608 at four dosage levels (10–40 mg/kg, s.c.) were administered. Figure 12, C and D, show the area under the

collected for one rat in the GZ-11608 group owing to a faulty catheter and for three rats in the saline group owing to an insecure head mount; data following procedural interruptions were not included in the analysis. * $P < 0.05$ compared with the saline group on the respective session; two-way mixed-factor ANOVA; $n = 3$ –6 rats/group.

Fig. 12. Pharmacokinetics were assessed following oral gavage or intravenous administration (A) and subcutaneous administration (B) in 15% Kolliphor-EL/85% saline. Dose-dependent pharmacokinetics following subcutaneous administration was conducted to assess the effect on AUC (C) and clearance (D). Linear regression was conducted to estimate whether the slope was significantly nonzero. $n = 6$ –9 rats/experiment.

concentration-time curves and clearance, respectively. Kinetic parameter estimates demonstrate that GZ-11608 exhibits linear kinetics with dose-proportional AUC increases ($P = 0.002$, runs test) and a constant clearance. Detailed pharmacokinetic parameters of individual experiments, estimated via noncompartmental modeling, are provided in Table 1.

Brain Biodistribution. The distribution of GZ-11608 into brain appears to be extensive. Following subcutaneous dosing, the apparent partition constant estimated by the ratio of the brain and plasma AUC_{0–12 hours} is approx. 21. The T_{max} is at approx. 1 hour after GZ-11608 (30 mg/kg, s.c.) administration, and following this time point the concentrations decrease at the same rate in brain as in plasma with an apparent half-life of 3 hours (Fig. 13). The rapid increase in brain concentrations, compared with plasma concentrations, suggests that GZ-11608 binds to brain tissues, but once plasma concentration reaches its peak and distribution equilibrates, the plasma clearance drives elimination from the brain.

Discussion

The current drug discovery program probed VMAT2 as a therapeutic target for identification of medications for MUD. Although a previous lead, GZ-793A, showed VMAT2 potency, selectivity, and efficacy, without the development of tolerance (Horton et al., 2011b; Alvers et al., 2012; Meyer et al., 2013; Nickell et al., 2017), it had potential cardiotoxicity (Nickell et al., 2017). Structural modification aimed at eliminating hERG affinity resulted in GZ-11610 and GZ-11608. GZ-11610 exhibited 9 nM K_i and 1090-fold selectivity for VMAT2 over

hERG, and specifically decreased methamphetamine sensitization (Lee et al., 2018), revealing advantages of this structural scaffold. In the current study, GZ-11608 potentially inhibited VMAT2 ($K_i = 25$ nM), with >100-fold selectivity at VMAT2 over nAChRs and hERG, and importantly, decreased methamphetamine sensitization, methamphetamine self-administration, and reinstatement, without itself exhibiting intrinsic reinforcing properties.

Interaction of methamphetamine with the dopamine system underlies its abuse liability (Koob and Volkow, 2016). Methamphetamine increases cytosolic dopamine concentration by releasing vesicular dopamine ($EC_{50} = 9$ – 15 μ M, $E_{max} = 90\%$) and inhibiting vesicular uptake ($K_i = 2.5$ μ M) (Nickell et al., 2010; Horton et al., 2013; current study). At VMAT2, methamphetamine inhibits uptake approx. 4-fold more potently than it evokes release. In contrast, GZ-11608 inhibits uptake 25-fold more potently than it evokes release ($EC_{50} = 0.62$ μ M, $E_{max} = 77\%$). GZ-11608 releases vesicular dopamine 23-fold more potently and inhibits uptake at VMAT2 100-fold more potently than methamphetamine. Moreover, the current study shows that GZ-11608 inhibits methamphetamine-evoked vesicular dopamine release and shifts rightward by 100-fold the methamphetamine concentration-response curve, with no change in E_{max} . The Schild slope not being different from unity suggests a competitive mechanism, such that GZ-11608 and methamphetamine appear to act at the same site on VMAT2.

Although most compounds known to interact with VMAT2 both release and inhibit its function, they are categorized as either releasers (substrates) or inhibitors on the basis of relative E_{max} (Partilla et al., 2006). Generally, compared with inhibitors, releasers have greater vesicular dopamine release

TABLE 1

Pharmacokinetic parameter estimates by noncompartmental analyses determined following intravenous, oral gavage, and subcutaneous dosing

Route	Dose	Parameters					
		$t_{1/2}$	T_{max}	C_{max}	$AUC_{0 \rightarrow \infty}$	CL or CL/F	F
	<i>g/kg</i>	<i>h</i>	<i>h</i>	<i>ng/ml</i>	<i>h*ng/ml</i>	<i>ml/h per kilogram</i>	<i>%</i>
Intravenous	5	1.2	—	—	1214.5	861.6	—
Oral gavage	40	2.5	0.9	54.0	345.8	56,169	3.6
Subcutaneous	10	4.5	1.0	425.6	2310.1	4328.8	95.1
Subcutaneous	10	3.2	1.0	435.8	2004.7	4988.3	82.5
Subcutaneous	17	2.3	1.0	595.5	3599.4	4723.0	87.2
Subcutaneous	17	2.8	2.0	515.7	3243.1	5241.8	78.5
Subcutaneous	30	2.6	1.0	1579.1	9781.6	3067.0	134.2
Subcutaneous	30	3.7	3.0	2686.7	8137.7	3686.5	111.7
Subcutaneous	30	2.3	6.1	780.3	4625.8	6485.3	63.5
Subcutaneous	45	2.9	1.0	1487.3	10,359.0	4344.0	94.8

E_{max} values. Classified as a releaser, methamphetamine releases 65% of preloaded dopamine and 90% of tyramine. In contrast, tetrabenazine and reserpine, classified as inhibitors, release dopamine and tyramine by 45% and 50%, respectively (Partilla et al., 2006). Our work shows that methamphetamine releases 90% of dopamine, whereas tetrabenazine and reserpine release 49% and 28%, respectively (current study; Horton et al., 2013). Since GZ-11608 and GZ-793A release 77% and 86%, respectively, they would probably be classified as releasers. Moreover, the current study employed classic pharmacological methods to determine mechanism of action (Kenakin, 1997). Schild analysis revealed that GZ-11608 and tetrabenazine competitively inhibit methamphetamine at VMAT2, whereas GZ-793A exhibited allosteric inhibition (current study; Horton et al., 2013).

The in vitro neurochemical effects of GZ-11608 translated to in vivo behavioral efficacy. GZ-11608 specifically decreased (>50%) methamphetamine sensitization following repeated administration. Methamphetamine sensitization reflects dynamic brain changes associated with MUD (Robinson and Berridge, 2008; London et al., 2015). Interestingly, the *R*-enantiomer GZ-11610 lacked specificity following subcutaneous administration, reducing activity in nonsensitized saline controls at doses that decreased methamphetamine sensitization (Lee et al., 2018).

Methamphetamine self-administration is considered the gold standard animal model of MUD (Panlilio and Goldberg, 2007; Mews and Calipari, 2017). GZ-11608 dose dependently decreased methamphetamine self-administration. Behavioral efficacy was found within the maximal

concentration-exposure window, as indicated by the dose-dependent linear pharmacokinetics, including similar T_{max} values across doses. Tolerance to GZ-11608's efficacy did not develop in the methamphetamine self-administration assay. Further, GZ-11608 specifically decreased methamphetamine self-administration when given across repeated injections. Although acute high doses of GZ-11608 (10–30 mg/kg) decreased food-maintained responding by approx. 20%, this effect was not significant. Moreover, repeated high-dose (30 mg/kg) administration significantly decreased food-maintained responding, but tolerance developed to this effect after five sessions. Different results between acute and repeated studies on food-maintained responding using the 30 mg/kg dose of GZ-11608 may be explained by the escalating-dose design of the acute study, such that some tolerance may have developed across the incrementing 1–17 mg/kg doses given prior to the 30 mg/kg dose of GZ-11608. Interestingly, during the post-treatment sessions after repeated administration, an apparent increase (10%–40%) above baseline in food-maintained responding was observed. Since MUD is often associated with decreased body weight (Sommers et al., 2006), increases in food intake may be a beneficial side effect.

MUD is characterized by high relapse rates with approx. 90% relapsing within 5 years (Wang et al., 2012; Brecht and Herbeck, 2014). Preclinical models characterize relapse using both surmountability and reinstatement assays. Prevention of a lapse developing into a relapse was evaluated by determining if higher methamphetamine doses could surmount the inhibitory effect of GZ-11608 on methamphetamine self-administration. GZ-11608 produced a downward and rightward shift of the dose-response curve for methamphetamine self-administration. Thus, increasing the methamphetamine dose did not surmount the GZ-11608-induced decrease in responding for methamphetamine. Notably, GZ-11608 tended to suppress responding for saline infusions compared with the no treatment condition, suggesting a nonspecific suppressant effect. However, the locomotor data do not support this interpretation, as GZ-11608 did not decrease activity. An alternative explanation is that GZ-11608 accelerated the within-session rate of extinction that occurred when saline was substituted for methamphetamine. Moreover, GZ-11608 dose dependently blocked both cue- and methamphetamine-induced reinstatement of methamphetamine seeking, supporting its potential utility in preventing relapse to methamphetamine seeking.

Decreases in methamphetamine self-administration and reinstatement may be attributable to reinforcing effects of

Fig. 13. Plasma and brain concentrations following GZ11608 (30 mg/kg, s.c.) administration. Terminal sampling was used to obtain plasma and brain. $n = 3$ rats/time point.

GZ-11608, such that it acts as a substitute for methamphetamine. Consistent with this possibility, GZ-11608 tended, although not significantly, to increase locomotor activity in the control group repeatedly injected with saline. However, GZ-11608 did not substitute for methamphetamine in the self-administration assay and did not engender self-administration in drug naive animals. The lack of reinforcing effects of GZ-11608 was not the result of faulty cannula, since availability of methamphetamine resulted in a return to maintenance levels of responding. Thus, GZ-11608 appears to decrease methamphetamine self-administration by inhibiting the reinforcing effect of methamphetamine, rather than by producing reinforcement itself. The lack of intrinsic reinforcing effects of GZ-11608 and the prediction of low abuse liability are consistent with its >200-fold selectivity for VMAT2 over DAT, with DAT inhibition being most closely associated with abuse liability (Seeman and Lee, 1975; Stathis et al., 1995).

Methamphetamine is well known to deplete striatal dopamine content (Bowyer et al., 1992, 1994). In contrast, GZ-11608 neither reduces dopamine content nor exacerbates the methamphetamine-induced decrease in content. Thus, during a relapse event when both GZ-11608 and methamphetamine may be onboard, no additional dopaminergic neurotoxicity would be predicted to occur. In contrast, tetrabenazine, a classic and reversible VMAT2 inhibitor with only 2-fold lower affinity for DAT, exacerbates methamphetamine-induced dopamine depletion (Kenney and Jankovic, 2006; Guay, 2010). Importantly, at low doses, tetrabenazine increases responding for methamphetamine, whereas at high doses responding for methamphetamine decreases; however, responding for food also decreases, indicating a lack of specificity (Meyer et al., 2011). Thus, relative to this classic VMAT2 inhibitor, GZ-11608 has considerable advantages as a potential MUD therapeutic.

Several lead compounds, i.e., lobeline, lobelane, GZ-793A, and GZ-11608, have been identified from our iterative drug discovery program (Harrod et al., 2001, 2003; Neugebauer et al., 2007; Meyer et al., 2011; Alvers et al., 2012; Beckmann et al., 2012; Horton et al., 2013; current study). VMAT2 affinity has increased, reaching the low nanomolar range with GZ-793A and GZ-11608. Schild regression on methamphetamine-evoked vesicular dopamine release revealed a surmountable allosteric mechanism for GZ-793A and an orthosteric mechanism for GZ-11608. Generally, the leads exhibit good selectivity for VMAT2 over DAT, do not exacerbate methamphetamine-induced striatal depletion, and have behavioral efficacy and specificity decreasing methamphetamine self-administration, without decreasing food-maintained responding. Interestingly, lobeline and GZ-793A produced downward shifts of the methamphetamine self-administration dose-response curve, whereas GZ-11608 produced a downward and rightward shift, suggesting potentially different underlying mechanisms. Nevertheless, the GZ-11608-induced decrease in methamphetamine self-administration was not surmounted by increasing the unit dose of methamphetamine. Although tolerance developed to lobelane's efficacy in decreasing methamphetamine self-administration, this was not the case for lobeline, GZ-793A and GZ-11608. Although lobeline was not efficacious in decreasing reinstatement of methamphetamine-seeking behavior, GZ-793A and GZ-11608 showed efficacy. However, since GZ-793A was eliminated as a potential therapeutic for MUD

owing to its potential cardiotoxicity, these new results advance GZ-11608 as a potential therapeutic as a result of its ability to specifically decrease methamphetamine self-administration and reinstatement. Moreover, GZ-11608 does not have intrinsic reinforcing properties and is expected to have low abuse liability.

Brain penetration of GZ-11608 appears to be exceedingly high with an estimated partition that is 20-fold higher than plasma. This is consistent with the low molecular weight and lipophilic structure of the compound, which probably promote brain tissue binding. As is typical with most drugs that highly penetrate the blood-brain barrier, it is the plasma unbound concentration that drives biodistribution and as such unbound fractions equilibrate rapidly between the two compartments (Tuntland et al., 2014). Once GZ-11608 reaches equilibrium, it is cleared from the brain at the same rate as it is from the plasma.

In summary, GZ-11608, a potent and selective VMAT2 inhibitor, specifically decreases methamphetamine reinforcement, and tolerance does not develop to its efficacy. Methamphetamine does not surmount the GZ-11608-induced decrease in responding for methamphetamine, and GZ-11608 decreased both cue- and methamphetamine-induced reinstatement of methamphetamine-seeking behavior. GZ-11608 also appears to have low abuse liability. With the exception of its low oral bioavailability, these preclinical findings suggest that GZ-11608 has good efficacy and potential as a therapeutic for MUD.

Acknowledgments

We acknowledge Dr. Joshua S. Beckmann for providing advice regarding statistical analyses. We also thank Agripina Deaciuc for technical assistance with the neurochemical experiments, and Dr. Jamie Horn, Emily Denehy, Karen Jackson, Scott Kinison, Seth Mayfield, and Savannah Schlueter for technical assistance with the pharmacokinetic and behavioral experiments.

Authorship Contributions

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