Metabotropic glutamate receptor 5 negative allosteric modulators as novel tools for in vivo investigation

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1. **Supplemental table S1**. Off-target binding profile of 7i (MFZ 10-7).

Assay Type	Binding inhibition	
	100 nM	10,000 nM
Acetylcholine Esterase		
Adenosine, Non-selective		
Adrenergic, Alpha-1, Non-selective		
Adrenergic, Alpha-2, Non-selective		
Adrenergic, Beta-1 (h)		
Adenosine, Non-selective		
Angiotensin II, AT1 (h)		
Angiotensin II, AT2, Central		
Bradykinin, BK2		
Calcium Channel, Type L (Benzothiazepine Site)		
Calcium Channel, Type L (Dihydropyridine Site)		
Calcium Channel, Type N		
Cannabinoid, CB1 (hr)		
Cannabinoid, CB2 (hr)		
Cholecystokinin, CCKA		
Cholecystokinin, CCKB		
Choline Acetyl Transferase, ChAT		
CRF, Non-Selective		
Dopamine, D4.2 (hr)		
Endothelin, ETA (h)		
Endothelin, ETB (h)		
Estrogen (h)		
GABA-A, Agonist Site		
GABA-A, BDZ, Alpha-1 Site		

GABA-B		
Galanin, Non-Selective		
Glucocorticoid (h)		
Glutamate, AMPA Site		
Glutamate, Kainate Site		
Glutamate, NMDA, Glycine [Strychnine-insensitive]		
Glutamate, mGluR1		
Glutamate, mGluR5	**91.49%**	**99.25%**
Glutamate, NMDA, Agonist Site		
Glutamate, NMDA, PCP Site		
Glutamic Acid Decarboxylase, GAD		
Glycine, Strychnine-sensitive Site		
Histamine, H1		
Histamine, H2		
Histamine, H3		
LTB4, Agonist (h)		
LTD4 (CysLT1)		
MAO-A, peripheral		
MAO-B, peripheral	**0.20%**	**75.80%**
Muscarinic, Non-selective, Central		
Muscarinic, Non-selective, Peripheral		
Muscarinic, M1 (hr)		
Muscarinic, M2 (hr)		
Neurokinin, NK1		
Neurokinin, NK2, NKA (hr)		
Neurokinin, NK3, NKB		
Nicotinic [α-Bungarotoxin Insensitive, Neuronal] (h)		
Nicotinic [α-Bungarotoxin Sensitive, Muscle-Type] (H)		
Nitric Oxide Synthase, NOS (neuronal-binding)		
Opiate, Kappa 1		
Opiate, Mu (h)		
Oxytocin		
Platelet Activating Factor, PAF		
Potassium Channel, ATP Sensitive (KATP)		
Potassium Channel, Ca2+ Activated, VI		
Sodium channel, Site 2		
Thyrotropin Releasing Factor, TRH		
Testosterone, Cytosolic (h)		
Thromboxane, TXA2 (h)	**12.82%**	**66.36%**
Vasoactive Intestinal Peptide, Non-selective		
Vasopressin, V1		

*Gray fill indicates no detectable binding at given concentration of **7i**.*

2. Experimental methods

2.1 Chemistry

Reaction conditions and yields were not optimized and spectroscopic data and yields refer to the free base unless otherwise described in each compound. Flash chromatography was performed using silica gel (EMD Chemicals, Inc.; 230-400 mesh, 60 Å). ^{1}H and ^{13}C NMR spectra were acquired using a Varian Mercury Plus 400 spectrometer. Chemical shifts are reported in parts-per-million (ppm) and referenced according to deuterated solvent for ^{1}H spectra ($CDCl_3$, 7.26), ^{13}C spectra ($CDCl_3$, 77.2). Infrared spectra were recorded as a KBr pellet using a Perkin-Elmer Spectrum RZ I FT-IR spectrometer. Gas chromatography-mass spectrometry (GC/MS) data were acquired using an Agilent Technologies (Santa Clara, CA) 6890N GC equipped with an HP-5MS column (cross-linked 5% PH ME siloxane, 30 m × 0.25 mm i.d. × 0.25 μm film thickness) and a 5973 mass-selective ion detector in electron-impact mode. Ultrapure-grade helium was used as the carrier gas at a flow rate of 1.2 mL/min. The injection port and transfer line temperatures were 250 and 280 ℃, respectively, and the oven temperature gradient used was as follows: the initial temperature (100 ℃) was held for 3 min and then increased to 295 ℃ at 15 ℃/min over 13 min, and finally maintained at 295 ℃ for 10 min. Combustion analysis was performed by Atlantic Microlab, Inc. (Norcross, GA) and agrees within 0.5% of calculated values. Melting point determination was conducted using a Thomas-Hoover melting point apparatus and the thermometer is uncorrected. Anhydrous solvents were purchased from Aldrich or J. T. Baker and were used without further purification, except for tetrahydrofuran, which was freshly distilled from sodium-benzophenone ketyl. All other chemicals and reagents were purchased from Aldrich Chemical Co., Combi-Blocks, Matrix Scientific, Lancaster Synthesis, Inc. (Alfa Aesar) and AK Scientific, Inc. The final products were converted into HBr salts, typically by treating the free base with methanolic HBr followed by precipitation from a combination of organic solvents. On the basis of NMR, GC-MS, and combustion data, all final compounds are >95% pure. Preparation of 2-methyl-6-trimethylsilanylethynylpyridine **5** was according the literature procedures.[1] Compounds **1** and **2** were purchased from Tocris (Ellisville, MO).

2.1.1 General Procedure A for the synthesis of aryltrimethylsilylacetylene

To a solution of aryl bromide in degassed E_3N (3 mL / mmol of ArBr) was added trimethylsilylacetylene **4** (1.1 eq), CuI (10%), and $Pd(PPh_3)Cl_2$ (5%). The resulting solution was stirred at RT overnight under argon. The black solution was then filtered over Celite and the filtrate was concentrated under reduced pressure. The residue was diluted with H_2O, extracted with EtOAc. The combined organic layers were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography to give the pure products **5** or **6a-c**.

2.1.2 General Procedure B for the Sonagashira coupling

To the mixture of 2-methyl-6-[(trimethylsilyl)ethynyl]pyridine **5** or aryltrimethylsilylacetylene **6a-c** (1 eq), aryl bromide (1.2 eq), CuI (20%) and $Pd(PPh_3)_4$ (5%) in DMF (5mL DMF /1 mmol scale reaction) was added Bu_4NF (1 eq, 1.0 M solution in THF) dropwise at 70 ℃ under Argon. After the addition, the mixture was stirred at this temperature overnight. The mixture was then filtered through Celite, DMF was removed under reduced pressure, and the residue was diluted with H_2O and extracted with EtOAc. The combined organic layers were dried ($MgSO_4$) and concentrated. The residue was purified by flash column chromatography to afford the pure product.

2.1.3 Synthesis of 3-[(Trimethylsilyl)ethynyl]benzonitrile (6a)

This compound was prepared from trimethylsilylacetylene **4** (6.67 g, 68.2 mmol) and 3-bromobenzonitrile (10.2 g, 56 mmol) according to procedure **A** and purified by column chromatography, eluting with hexane/EtOAc(10/1) to afford pure product (11.07 g) as a light brown oil in 99% yield, which solidified to a light brown solid after standing at room temperature. Mp 41.5-42.5 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.26(s, 9H), 7.42(dt, J=0.8, 8.0 Hz, 1H), 7.58(dt, J=1.2, 8.0 Hz, 1H), 7.66(dt, J=1.2, 8.0 Hz, 1H), 7.74(m, 1H), ppm.

2.1.4 Synthesis of 3-Fluoro-5-[(trimethylsilyl)ethynyl]benzonitrile (6b; ZP 3-53)

This compound was prepared by following the general procedure **A** using 3-bromo-5-fluorobenzonitrile (0.8 g, 4 mmol), eluting with hexane/ethyl acetate (6:1) to give the product as a syrup (0.8 g) in 92% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (s, 1H), 7.19-7.10 (m, 2H), 0.07 (s, 9H), ppm; GC-MS (EI) *m/z* 217 (M^+).

2.1.5 Synthesis of 5-((Trimethylsilyl)ethynyl)nicotinonitrile (6c; ZP 3-59)

This compound was prepared by following the general procedure **A** using 3-bromo-5-cyanopyridine (0.37 g, 2 mmol), and purified by column chromatography eluting with hexane/ethyl acetate (6:1) to give a solid (0.4 g)

in 99% yield; Mp 92-94 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.78 (s, 1H), 8.00 (s 1H), 0.07 (s, 9H), ppm; GC-MS (EI) *m/z* 200 (M^+).

2.1.6 Synthesis of 6-cyano-2-(3-cyanophenylethynyl)pyridine (7a; MFZ 10-35)

This compound was prepared by the coupling of **6a** (400 mg, 2.0 mmol) and 2-bromo-6-cyanopyridine (439 mg, 2.4 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (1/1) to afford the pure product (442 mg) as light brown solid in 97% yield. Compound **7a** was recrystallized from methanol to give the product as white crystals. Mp 152-152 ℃ (dec). ^{1}H NMR(400 MHz, $CDCl_3$) δ 7.53(t, J=7.6 Hz, 1H), 7.68-7.72(m, 2H), 7.40(dd, J=1.2, 8.0 Hz, 1H), 7.83(dt, J=1.2, 8.0 Hz, 1H), 7.87-7.91(m, 2H), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 88.58, 88.78, 113.25, 116.42, 117.69, 123.03, 127.71, 129.58, 130.28, 132.80, 134.48, 135.43, 136.17, 137.60, 144.28, ppm; GC-MS (EI) (m/z): 229(M+); Anal. ($C_{15}H_7N_3$) for C, H, N.

2.1.7 Synthesis of 6-Hydroxymethyl-2-(3-cyanophenylethynyl)pyridine (7b; MFZ 10-43)

This compound was prepared by the coupling of **6a** (400 mg, 2.0 mmol) and 2-bromo-6-hydroxymethylpyridine (451 mg, 2.4 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (1/1.5) to afford the pure product (458 mg) as light brown solid in 98% yield. The HBr salt was made by adding HBr in methanol to the free base and recrystallized from methanol. Mp 178-180 ℃ (dec). ^{1}H NMR(400 MHz, $CDCl_3$) δ 4.81(s, 2H), 7.31(d, J=7.6 Hz, 1H), 7.47(d, J=8.8 Hz, 1H), 7.51(d, J=8.0 Hz, 1H), 7.66(dt, J=1.2, 7.6 Hz, 1H), 7.73, (t, J=8.0 Hz, 1H), 7.82(dt, J=1.2, 7.6 Hz, 1H), 7.87(s), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 64.30, 86.63, 90.46, 113.06, 117.90, 120.40, 123.87, 126.15, 129.43, 132.20, 135.30, 136.05, 137.03, 141.48, 160.13, ppm; GC-MS (EI) (m/z): 233(M-1); Anal. ($C_{15}H_{10}N_2O{\cdot}HBr$) for C, H, N.

2.1.8 Synthesis of 6-Methoxy-2-(3-cyanophenylethynyl)pyridine (7c; MFZ 10-21)

This compound was prepared by the coupling of **6a** (400 mg, 2.0 mmol) and 2-bromo-6-methoxypyridine (451 mg, 2.4 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (3/1) to afford the pure product (420 mg) as light grey solid in 90% yield. The HBr salt was made by adding HBr in methanol to the free base and recrystallized from EtOH. Mp 189.5-192 ℃ (dec). ^{1}H

NMR(400 MHz, $CDCl_3$) δ 3.98(s, 3H), 6.76(d, J=8.8 Hz, 1H), 7.16(d, J=7.2 Hz, 1H), 7.49(t, J=8.0 Hz, 1H), 7.57(dd, J=8.0, 7.6 Hz, 1H), 7.64(dt, J=1.2, 8.0 Hz, 1H), 7.80(dt, J=1.2, 8.4 Hz, 1H), 7.87(s), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 54.43, 86.64, 90.37, 113.04, 117.58, 120.94, 123.42, 126.67, 129.58, 132.13, 135.24, 136.30, 137.17, 141.76, 163.55, ppm; GC-MS (EI) (m/z): 233(M-1); Anal. ($C_{15}H_{10}N_2O \cdot HBr$) for C, H, N.

2.1.9 Synthesis of 6-acetyl-2-(3-cyanophenylethynyl)pyridine (7d; MFZ 10-37)

This compound was prepared by the coupling of **6a** (400 mg, 2.0 mmol) and 2-bromo-6-acetylpyridine (480 mg, 2.4 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (1.5/1) to afford the pure product (414 mg) as light brown solid in 84% yield. The HBr salt was made by adding HBr in methanol to the free base and recrystallized from EtOH. Mp 122.5-125 ℃. ^1H NMR(400 MHz, $CDCl_3$) δ 2.77(s), 7.53(t, J=8 Hz, 1H), 7.65-7.72 (m, 2H), 7.83-7.89(m, 2H), 7.92(m, 1H), 8.03(dd, J=1.2, 7.6 Hz, 1H), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 25.81, 86.79, 90.21, 113.11, 117.83, 121.20, 123.69, 127.56, 129.46, 130.65, 131.32, 132.36, 135.37, 136.07, 137.26, 142.00, 153.97, 199.54, ppm; GC-MS (EI) (m/z): 246 (M+); Anal. ($C_{16}H_{10}N_2O \cdot HBr$) for C, H, N.

2.1.10 Synthesis of 6-Fluoro-2-(3-cyanophenylethynyl)pyridine (7e; MFZ 10-20)

This compound was prepared by the coupling of **6a** (400 mg, 2.0 mmol) and 2-bromo-6-fluoropyridine (422 mg, 2.4 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (3/1) to afford the pure product (481 mg) in 93% yield. The HBr salt was made by adding HBr in methanol to the free base and recrystallized from 2-PrOH. Mp 178-181 ℃ (dec). ^1H NMR(400 MHz, $CDCl_3$) δ 6.97(ddd, J=0.8, 2.8, 8.4 Hz, 1H), 7.44(ddd, J=0.8, 2.8, 7.6 Hz, 1H), 7.51(t, J=8.0 Hz, 1H), 7.67(dt, J=1.2, 8.0 Hz, 1H), 7.79-7.87(m, 3H), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 87.38, 89.30, 110.15(d, J=36.6 Hz, C-F), 113.12, 117.82, 123.52, 124.91, 129.48, 132.42, 135.30, 136.08, 141.44, 161.84, 164.25, ppm; GC-MS(EI) (m/z): 222(M+); Anal. ($C_{14}H_7N_2F \cdot HBr$) for C, H, N.

2.1.11 Synthesis of 6-Ethyl-2-(3-cyanophenylethynyl)pyridine (7f; MFZ 10-26)

This compound was prepared by the coupling of **6a** (400 mg, 2.0 mmol) and 2-bromo-6-ethylypyridine (390 mg, 1.05 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (3/1) to afford the pure product (408 mg) as a light brown oil in 88% yield. ^1H NMR(400 MHz,

$CDCl_3$) δ 1.33(t, J=7.6 Hz, 3H), 2.87(q, J=7.6 Hz, 2H), 7.18(d, J=8.0 Hz, 1H), 7.38(d, J=7.6 Hz, 1H), 7.48(t, J=8.0 Hz, 1H), 7.60-7.66(m, 2H), 7.81(dt, J=1.2, 7.6 Hz, 1H), 7.87(m, 1H), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 14.05, 31.57, 85.90, 91.10, 112.95, 117.98, 124.17, 129.33, 129.48, 131.98, 132.06, 135.28, 136.05, 136.68, 141.78, 164.47, ppm; GC-MS (EI) (m/z): 232 (M+), 231(M-1); Anal. ($C_{16}H_{12}N_2$) for C, H, N.

2.1.12 Synthesis of 6-Butyl-2-(3-cyanophenylethynyl)pyridine (7g; MFZ 10-38)

This compound was prepared by the coupling of **6a** (400 mg, 2.0 mmol) and 2-bromo-6-butylpyridine (470 mg, 2.2 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (2/1), to afford the pure product (470 mg) as a light brown oil in 91% yield. ^{1}H NMR(400 MHz, $CDCl_3$) δ 0.94(t, J=7.2 Hz, 3H), 1.36-1.44(m, 2H), 1.68-1.75(m, 2H), 2.83 (t, J=8.0 Hz, 2H), 7.17(dd, J=0.8, 7.6 Hz, 1H), 7.40(dd, J=0.8, 8.0 Hz, 1H), 7.48(t, J=8.0 Hz, 1H), 7.60-7.65(m, 2H), 7.79-7.87(m, 2H), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 13.94, 22.52, 32.17, 38.26, 85.92, 91.08, 112.93, 117.98, 112.67, 114.18, 114.88, 128.46, 131.96, 135.28, 136.05, 136.54, 141.81, 163.46, ppm; GC-MS (EI) (m/z): 259 (M-1); Anal. ($C_{18}H_{16}N_2$) for C, H, N.

2.1.13 Synthesis of 6-Methyl-2-[(3-cyano-5-methoxyphenyl)ethynyl]pyridine (7h; MFZ 10-17)

This compound was prepared by the coupling of **5** (378 mg, 2.0 mmol) and 3-bromo-5-methoxybenzonitrile (509 mg, 2.4 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (1.5/1) to afford the pure product (481 mg) in 97% yield. The HBr salt was made by adding HBr in methanol to the free base and recrystallized from EtOH. Mp 158-161 ℃ (dec). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.60(s, 3H), 3.85(s, 3H), 7.13-7.15(m, 1H), 7.16(d, J=8.0 Hz, 1H), 7.33-7.35(m, 1H), 7.37(d, J=7.6 Hz, 1H), 7.45(t, J=1.2 Hz, 1H), 7.60(t, J=8.0 Hz, 1H), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 24.55, 55.75, 85.99, 90.53, 113.56, 117.88, 118.10, 121.60, 123.27, 124.61, 125.06, 127.56, 136.52, 141.73, 159.22, 159.54, ppm; GC-MS (EI) (m/z): 248(M+); Anal. ($C_{16}H_{12}N_2O$.HBr.1/2H_2O) for C, H, N.

2.1.14 Synthesis of 6-Methyl-2-[(3-cyano-5-fluorophenyl)ethynyl]pyridine (7i; MFZ 10-7)

This compound was prepared by the coupling of **5** (427.5 mg, 2.5 mmol) and 3-bromo-5-fluorobenznitrile (600 mg, 3.0 mmol) according to the general procedure **B**, and purified by column chromatography, eluting with hexane/EtOAc (3/1) to afford the pure product (557 mg) in 94% yield. The HBr salt was made by adding

HBr in methanol to the free base and recrystallized from EtOH. Mp 165-167.5 ℃ (dec). ^{1}H NMR(400 MHz, $CDCl_3$) δ 2.60(s, 3H), 7.18(d, J=7.6 Hz, 1H), 7.32-7.39(m, 2H), 7.52(dq, J=1.2, 8.8 Hz, 1H), 7.62(t, J=8.0 Hz, 1H), 7.67(t, J=1.2 Hz, 1H), ppm; ^{13}C NMR(100 MHz, $CDCl_3$) δ 24.59, 84.65, 91.96, 114.24, 119.25, 119.50, 123.37, 123.59, 124.78, 126.29, 131.51, 136.61, 141.36, 159.43, 160.66, 163.16, ppm; GC-MS (EI) (m/z): 236(M+); Anal. ($C_{15}H_9N_2F$·HBr) for C, H, N.

2.1.15 Synthesis of 6-Cyano-2-[(3-cyano-5-fluorophenyl)ethynyl]pyridine (7j; ZP 3-74)

This compound was prepared by following the general procedure **B**, coupling **6b** (0.44 g, 2 mmol) and 2-bromo-6-cyanopyridine (0.36 g, 2 mmol) at 70 ℃ for 3 h, and purified by column chromatography eluting with hexane/ethyl acetate (4:1) and ethyl acetate to give the product (0.3 g) in 61% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (m, 1H), 7.70 (m, 3H), 7.55 (m, 1H), 7.43 (m, 1H), ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.2, 160.7, 143.8, 137.7, 134.6, 131.6, 130.3, 128.0, 123.8, 123.5, 120.4, 120.1, 89.6, 87.2, ppm; ^{19}F NMR (376 MHz, $CDCl_3$) δ -108.7 ppm; GC-MS (EI) *m/z* 247 (M^+); The HBr salt was made by adding HBr in methanol to the free base and recrystallized from acetone; Mp 144-146 ℃ (dec); Anal. ($C_{15}H_6FN_3$·HBr·7/4H_2O) for C, H, N.

2.1.16 Synthesis of 6-Hydroxymethyl-2-[(3-cyano-5-fluorophenyl)ethynyl]pyridine (7k; ZP 3-77)

This compound was prepared by following the general procedure **B**, coupling **6b** (0.44 g, 2 mmol) and 2-bromo-6-hydroxymethylpyridine (0.38 g, 2 mmol) at 70 ℃ for 3 h, and purified by column chromatography eluting with hexane/ethyl acetate (4:1) and ethyl acetate to give the product (0.22 g) in 44% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (m, 1H), 7.69 (s, 1H), 7.54 (d, *J* = 9.2 Hz, 1H), 7.48 (d, *J* = 7.6 Hz, 1H), 7.38 (d, *J* = 7.6 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 1H), 4.81 (d, *J* = 5.6 Hz, 2H), 3.29 (m, 1H), ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.2, 141.0, 137.1, 131.5, 126.3, 123.6, 120.7, 119.7, 119.5, 114.5, 91.4, 85.3, ppm; ^{19}F NMR (376 MHz, $CDCl_3$) δ -109.3 ppm; GC-MS (EI) *m/z* 252 (M^+); The HBr salt was made by adding HBr in methanol to the free base and recrystallized from methanol; Mp 173-176 ℃ (dec); Anal. ($C_{15}H_9FN_2O$·HBr) for C, H, N.

2.1.17 Synthesis of 6-Methoxy-2-[(3-cyano-5-fluorophenyl)ethynyl]pyridine (7l; ZP 3-78)

This compound was prepared by following the general procedure **B**, coupling **6b** (0.22 g, 1 mmol) and 2-bromo-6-methoxypyridine (0.19 g, 1 mmol) at 68 ℃ for 3 h, and purified by column chromatography eluting with hexane/ethyl acetate (6:1 and 3:1) to give the product (0.2 g) in 79% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ

7.68 (s, 1H), 7.58 (m, 1H), 7.53 (d, *J* = 9.6 Hz, 1H), 7.36 (d, *J* = 7.6 Hz, 1H), 7.16 (d, *J* = 7.6 Hz, 1H), 6.78 (d, *J* = 7.6 Hz, 1H), 3.97 (s, 3H), ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.1, 138.9, 138.6, 131.5, 123.5, 123.3, 121.2, 119.4, 119.2, 112.3, 92.0, 84.4, 65.9, ppm; ^{19}F NMR (376 MHz, $CDCl_3$) δ -109.5 ppm; GC-MS (EI) m/z 252 (M^+); The HBr salt was made by adding HBr in methanol to the free base and recrystallized from methanol; Mp 207-209 ℃ (dec); Anal. ($C_{15}H_9FN_2O$·4/5HBr) for C, H, N.

2.1.18 Synthesis of 6-Acetyl-2-[(3-cyano-5-fluorophenyl)ethynyl]pyridine (7m; ZP 3-85)

This compound was prepared by following the general procedure **B**, coupling **6b** (0.43 g, 2 mmol) and 2-bromo-6-acetylpyridine (0.35 g, 2 mmol) at 70 ℃ for 3 h, and purified by column chromatography eluting with hexane/ethyl acetate (6:1 and 1:1) to give a solid (0.4 g) in 75% yield; Mp 135-136 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (d, *J* = 7.6 Hz, 1H), 7.88 (m, 1H), 7.72 (s, 1H), 7.71 (d, *J* = 8.8 Hz, 1H), 7.57 (d, *J* = 9.2 Hz, 1H), 7.41 (d, *J* = 8.0 Hz, 1H), 2.77 (s, 3H), ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.2, 154.0, 141.6, 137.3, 131.6, 130.7, 125.9, 123.7, 123.4, 121.5, 119.9, 119.6, 114.5, 91.2, 85.5, 25.8, ppm; ^{19}F NMR (376 MHz, $CDCl_3$) δ -109.2 ppm; GC-MS (EI) m/z 264 (M^+); Anal. ($C_{16}H_9FN_2O$ ·1/4H_2O) for C, H, N.

2.1.19 Synthesis of 6-Fluoro-2-[(3-cyano-5-fluorophenyl)ethynyl]pyridine (7n; ZP 3-86)

This compound was prepared by following the general procedure **B**, coupling **6b** (0.43 g, 2 mmol) and 2-bromo-6-fluoropyridine (0.4 g, 2 mmol) at 70 ℃ for 4 h, and purified by column chromatography eluting with hexane/ethyl acetate (6:1 and 1:1) and ethyl acetate to give a solid (0.3 g) in 63% yield; Mp 134-135 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (s, 1H), 7.93 (m, 1H), 7.63 (m, 1H), 7.47 (d, *J* = 8.4 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 6.99 (d, *J* = 9.2 Hz, 1H), ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.2, 151.0, 150.9, 143.9, 143.8, 131.2, 123.2, 123.0, 119.5, 119.3, 110.0, 109.6, 88.7, 88.1, ppm; ^{19}F NMR (376 MHz, $CDCl_3$) δ -109.2, -64.6, ppm; GC-MS (EI) m/z 240 (M^+); Anal. ($C_{14}H_6F_2N_2$) for C, H, N.

2.1.20 Synthesis of 6-[(5-Cyanopyridin-3-yl)ethynyl]picolinonitrile (7o; ZP 3-80)

This compound was prepared by following the general procedure **B**, coupling **6c** (0.31 g, 1.6 mmol) and 2-bromo-6-cyanopyridine (0.28 g, 1.6 mmol) at 70 ℃ for 4 h, and purified by column chromatography eluting with hexane/ethyl acetate (1:1) to give a solid (0.2 g) in 56% yield; Mp 184-186 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.01 (s, 1H), 8.89 (s, 1H), 8.16 (m, 1H), 7.92 (m, 1H), 7.78-7.72 (m, 2H), ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ

155.4, 151.8, 143.6, 141.7, 137.8, 134.6, 130.3, 128.1, 119.4, 116.3, 115.5, 110.1, 91.9, 85.0, ppm; GC-MS (EI) m/z 230 (M^+); Anal. ($C_{14}H_6N_4 \cdot 1/3H_2O$) for C, H, N.

2.1.21 Synthesis of 6-Hydroxymethyl-2-[(5-cyanopyridin-3-yl)ethynyl]pyridine (7p; ZP 3-89)

This compound was prepared by following the general procedure **B**, coupling **6c** (0.3 g, 1.6 mmol) and 2-bromo-6-hydroxymethylpyridine (0.28 g, 1.6 mmol) at 70 ℃ overnight. The product precipitated from MeOH (0.24 g) in 68% yield; The HBr salt was made by adding HBr in methanol to the free base and recrystallized from CH_2Cl_2; Mp 203-205 ℃ (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.63-7.40 (m, 6H), 3.27 (m, 2H), ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.7, 138.9, 134.5, 134.0, 133.9, 132.5, 132.0, 129.3, 129.2, 129.1, 105.0, 91.9, 84.1, 63.9, ppm; Anal. ($C_{14}H_9N_3O \cdot 3HBr \cdot 2H_2O$) for C, H, N.

2.1.22 Synthesis of 6-Methoxy-2-[(5-cyanopyridin-3-yl)ethynyl]pyridine (7q; ZP 4-4)

This compound was prepared by following the general procedure **B**, coupling **6c** (0.37 g, 1.8 mmol) and 2-bromo-6-methoxypyridine (0.22 mL, 1.8 mmol) at 65 ℃ overnight, and purified by column chromatography eluting with hexane/ethyl acetate (6:1) and ethyl acetate to give the product (0.12 g) in 28% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.98 (s, 1H), 8.83 (s, 1H), 8.13 (s, 1H), 7.60 (d, J = 7.6 Hz, 1H), 7.57 (s, 1H), 7.19 (d, J = 7.2 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 3.99 (s, 3H), ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.3, 155.6, 151.3, 141.7, 138.9, 121.5, 116.0, 112.8, 94.6, 82.5, 54.0 ppm; GC-MS (EI) m/z 234 (M^+); The HBr salt was made by adding HBr in methanol to the free base and recrystallized from acetone and methanol; Mp 195-196 ℃ (dec); Anal. ($C_{14}H_9N_3O \cdot 2HBr \cdot 6/5H_2O$) for C, H, N.

2.1.23 Synthesis of 5-Fluoro 3-[(6-cyanopyridin-2-yl)ethynyl]benzamide (7r; ZP 3-55)

This compound was prepared by following the general procedure **B**, coupling **6b** (0.44 g, 2 mmol) and 2-bromo-6-cyanopyridine (0.36 g, 2 mmol) at 72 ℃ overnight, and purified by column chromatography eluting with hexane/ethyl acetate (4:1) and ethyl acetate to give the product (0.3 g) in 57% yield; ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.1, 161.2, 143.7, 139.8, 138.0, 137.9, 133.9, 131.9, 129.5, 128.0, 122.9, 117.5, 116.8, 88.9, 79.7, ppm; ^{19}F NMR (376 MHz, $CDCl_3$) δ -110.7 ppm; GC-MS (EI) *m/z* 265 (M^+); The HBr salt was made by adding HBr in methanol to the free base and recrystallized from methanol; Mp 154-156 ℃ (dec); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.17-8.08 (3 m, 3H), 8.00 (m, 2H), 7.74 (m, 2H), 7.66 (s, 1H), ppm; Anal. ($C_{15}H_8FN_3O \cdot HBr$) for C,

H, N.

2.1.24 Synthesis of 3-[(6-Cyanopyridin-2-yl)ethynyl]benzamide (7s; ZP 3-69)

This compound was prepared by following the general procedure **B**, coupling **6a** (0.4 g, 2 mmol) and 2-bromo-6-cyanopyridine (0.37 g, 2 mmol) at 85 °C overnight, and purified by column chromatography eluting with hexane/ethyl acetate (6:1) and ethyl acetate to give the product (0.1 g) in 20% yield; GC-MS (EI) *m/z* 247 (M^+). The HBr salt was made by adding HBr in methanol to the free base and recrystallized from ethyl acetate; Mp 191-193 °C (dec); ^{1}H NMR (400 MHz, CD_3OD) δ 8.10 (m, 4H), 7.79 (d, J = 8.4 Hz, 1H), 7.65 (m, 2H), ppm; ^{13}C NMR (100 MHz, CD_3OD) δ 165.8, 161.2, 144.3, 132.6, 129.5, 127.5, 112.6, 109.8, 97.1, 88.5, 79.3, ppm; IR (thin film) 1710 cm^{-1}; Anal. ($C_{15}H_9N_3O{\cdot}2HBr\cdot 1/4C_3H_6O$) for C, H, N.

2.2 *In vitro* pharmacology

2.2.1 Radioligand binding assay

Binding at mGluR5 in rat brain membranes was determined by an adaptation of previously described methods.[2] Brains from male Sprague–Dawley rats weighing 200–225 g (Taconic Farms, Germantown, NY) were removed, and rapidly frozen. Membranes were prepared from whole brain minus the cerebellum by homogenizing tissue in 20 volumes (w/v) of cold assay buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.4 at 25 °C) using a Brinkman Polytron and centrifuged at 50,000 g for 10 min at 4 °C. The resulting pellet was re-suspended in cold assay buffer, re-centrifuged, and re-suspended in buffer to a concentration of 75 mg/mL. Ligand binding experiments were conducted in glass assay tubes containing 0.5 mL of buffer at room temperature for 60 minutes. Each reaction contained 4 nM [^{3}H]**1** (American Radiolabeled Chemicals, St. Louis, MO), 7.5 mg of brain tissue (original wet weight), and varying concentrations of test compounds. Nonspecific binding was determined using 10 μM **1**. Incubations were terminated by rapid filtration through Whatman GF/B filters, presoaked in 0.5% polyethylenimine, using a Brandel R48 filtering manifold (Brandel Instruments Gaithersburg, MD). The filters were washed thrice with 3 mL of cold assay buffer and transferred to scintillation vials. Beckman Ready Value (3.0 mL) was added, and the vials were counted using a Beckman 6000 liquid scintillation counter (Beckman Coulter Instruments, Fullerton, CA). Each compound was tested in full dose-

response curves, with test compound concentrations at half-log units ranging from 10 pM to 100 μM final concentration, each performed in triplicate, for competition against binding of [^{3}H]**1**. IC_{50} values were determined from at least three independent experiments.

2.2.2 *In vitro* functional assay

Novel mGluR5 NAMs were evaluated for functional activity *in vitro* using the IP-One ELISA assay (Cisbio US, Bedford, MA), a competitive immunoassay that can measure the production of D-*myo*-inositol 1 phosphate (IP1), a degradation product of IP3, a second messenger of $G_q\alpha$ signaling. The assay utilizes an anti-IP1 monoclonal antibody (Anti-IP1 Mab) and IP1-Horse-Radish Peroxidase (IP1-HRP) conjugate to measure accumulated IP1 levels in the presence of LiCl. In HEK293 cells stably expressing rat mGluR5, $G_q\alpha$-mediated IP3 production can be stimulated with quisqualic acid (QA), reversible using mGluR5 NAMs; inverse agonists will reduce baseline IP3 production. For each assay, test compounds were dissolved in 30% DMSO and water to a concentration of 100 μM and serially diluted in 1X stimulation buffer. The final concentrations of test compounds ranged from 10 μM to 10 pM, in 0.3% DMSO and 1X stimulation buffer. QA was dissolved in 1X stimulation buffer to final concentrations from 100 μM to 100 pM; 1 μM QA was used as the agonist to test antagonism capabilities of mGluR5 NAMs. A six-point dose response curve for each test compound as well as vehicle-only and QA-only controls, each point run in duplicate, allowed each individual dose response to be contained within one 16-well IP-One ELISA test strip.

HEK293 cells stably expressing human mGluR5 were grown in DMEM with 20 mM HEPES, 2 mM L-glutamine, 10% FBS, 1 mM sodium pyruvate, 1X non-essential amino acids, 1X antibiotic/antimycotic, and 500 μg/mL G418 and kept in an incubator at 37° C and 5% CO_2. On the day prior to the assay, cells were plated in 24-well plates at 400,000 cells/well in 500 μl plating media (DMEM with 20 mM HEPES, 10% dialyzed FBS, 1 mM sodium pyruvate). On the day of the assay, cell media was aspirated and 100 μl of test compound in stimulation buffer was added, followed by 100 μl of 2 μM QA in stimulation buffer. For inverse agonism tests, 200 μl of test compound in stimulation buffer was added to each well with no QA present. After 1 hour incubation at 37° C, 50 μl of 2.5% lysis reagent was added to each well and the plates were shaken at 200 rpm, for 30 minutes at 37° C. Next, 50 μl supernatant from each well was transferred to the ELISA plate, along

with 25 µl Anti-IP1 Mab and 25 µl IP1-HRP. The plate was covered and incubated for 3 hours at room temperature with shaking (200 rpm). After a wash step, 100 µl of the HRP substrate, TMB, was added to each well to reveal the enzymatic activity. The plate was covered and incubated for 30 minutes in a dark environment. The addition of 100 µl Stop Solution stabilized the enzymatic signal and each well's optical density was determined at wavelengths of 450 nm and 620 nm (Spectramax M5 reader and Softmax Pro 5.3 software, Molecular Devices, Sunnyvale, CA). Relative IP1 levels for each treatment were determined by subtracting the OD at 620 nm from the OD at 450 nm, averaging the duplicates, and normalizing to vehicle-only control values (presented as % inhibition / basal). IC_{50} values for inverse agonism were calculated from at least three independent experiments in the absence of QA.

2.3 *In vivo* pharmacology

2.3.1 Drugs

For behavioral tests, **1**, **7i**, and **7j** were dissolved in 2% ethanol, 10% Tween 80 and water. Compound **2** was dissolved in physiological saline.

2.3.2 Animals

Male Swiss Webster mice (Taconic Farms, Germantown, NY), approximately 8-12 weeks old and weighing 25-35 g at the time of testing, were housed 3-4 to a cage and given food and water *ad libitum*. All subjects were kept in plastic cages with pine sawdust bedding in a colony room maintained at 21 ± 1° C under a 12-h light/dark cycle (lights on 7:00 AM). All experiments were conducted during the light phase of the light/dark cycle, 11:00 AM to 5:00 PM, in a room that was separate from the housing room. All animals were given a minimum of seven days to equilibrate to the housing facility before any experiments were performed. Animal care procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, National Institute on Drug Abuse Intramural Research Program.

Mice were tested for anxiolytic activity in the open field test and light-dark box test on consecutive days. All drugs were administered via intraperitoneal (i.p.) injection at a dose of 10 mL/kg and the same drug and dose was received in each test. Mice were weighed prior to each test session in order to provide an accurate mg/kg dose. Lights in the testing facility were increased to the highest intensity and a white noise generator provided

background noise.

2.3.3 Open field test

For the assessment of open field locomotor activity, mice were tested alone in clear acrylic experimental chambers (40 cm × 40 cm × 40 cm). Arrays of infrared light sources were mounted on the outside of two perpendicular adjoining walls of the chambers, spaced 2.5 cm apart, with arrays of light-sensitive detectors mounted opposite (Omnitech Electronics, Columbus, OH). Mice were moved to the testing facility and allowed to equilibrate to the behavioral test room in the home cage for 30 minutes. A single i.p. injection of vehicle or drug was given and the mouse was returned to the home cage for an additional 15 minutes. A single mouse was placed in the corner of a novel open field and horizontal locomotion and vertical activity were recorded for 15 minutes. The open field was divided electronically into a grid to evaluate activity occurring in the center region; anxiolysis in the novel open field test is indicated by an increase in the time spent in the center region.

2.3.4 Light-dark box test

One day after the open field test, mice were tested in the light-dark box test. Mice were moved to the testing facility and allowed to equilibrate to the behavioral test room in the home cage for 30 minutes. A single i.p. injection of vehicle or drug was given and the mouse was returned to the home cage for an additional 15 minutes. The open field (described above) was divided into a well-lit compartment and a dark compartment by a black acrylic insert, opaque to white light but permissive of infrared beams, with a small opening at the center-bottom to allow movement between the two compartments; the insert divided the open field approximately 50:50 into light and dark compartments. A single mouse was placed, through the opening, into the dark side of the field and several behavioral measures were recorded for 15 minutes. Anxiolysis in the light-dark box test is indicated by an increase in the time spent in the light compartment and a greater number of entries into the light compartment.

2.4 Data analysis

Data for all *in vitro* and *in vivo* tests were analyzed using GraphPad Prism software version 5.00 (San Diego, CA).

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