Supplemental Info

Discovery of the first selective M_4 muscarinic acetylcholine receptor antagonists with *in vivo* anti-parkinsonian and anti-dystonic efficacy

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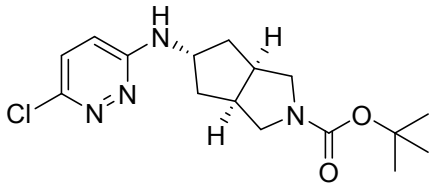
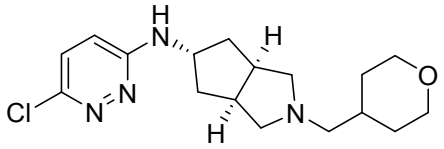


tert-butyl (3a*R*,5s,6a*S*)-5-((6-chloropyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (3). Tert-butyl (3a*R*,5s,6a*S*)-5-aminohexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (5.0 g, 22.1 mmol, 1 eq) and 3,6-dichloropyridazine (9.87 g, 66.3 mmol, 3 eq) were combined in tert-butanol (30 mL), and DIPEA (11.5 mL, 66.3 mmol, 3 eq) was added. The resulting solution was heated to 150 °C under microwave irradiation for 2 h, after which time the reaction mixture was concentrated under reduced pressure, and crude residue was purified by column chromatography (3-100% EtOAc in hexanes) to give the title compound as a white solid (4.87 g, 65%).

^{1}H-NMR (400 MHz, MeOD) δ 7.27 (d, *J* = 9.4 Hz, 1H), 6.87 (d, *J* = 9.4 Hz, 1H), 4.41 (p, *J* = 6.3 Hz, 1H), 3.55 (dd, *J* = 11.1, 8.0 Hz, 2H), 3.19 (dd, *J* = 11.4, 3.8 Hz, 2H), 2.90 – 2.80 (m, 2H), 1.90 – 1.92 (m, 2H), 1.89 – 1.81 (m, 2H), 1.46 (s, 9H).

^{13}C-NMR (101 MHz, MeOD) δ 159.3, 156.3, 146.8, 130.3, 120.5, 80.8, 53.7, 53.2 (*signal broadening is observed*) 42.3 (*signal broadening is observed*), 39.5, 28.8.

ES-MS $[M+H]^+$ = 283.2 (- t-butyl).

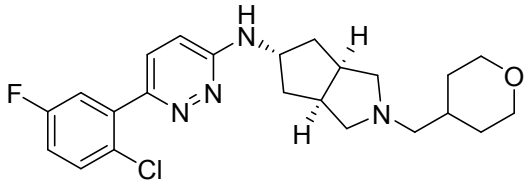


(3a*R*,5s,6a*S*)-*N*-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (5). Tert-butyl (3a*R*,5s,6a*S*)-5-((6-chloropyridazin-3-yl)amino)hexahydrocyclopenta[c]pyrrole-2(1H)-carboxylate (**3**) (4.86 g, 14.3 mmol, 1 eq) was dissolved in 1,4-dioxane (70 mL) and MeOH (20 mL), and 4M HCl in dioxanes solution (50 mL) was added dropwise. The resulting solution was stirred at r.t. for 1 h, after which time solvents were concentrated under reduced pressure to give the HCl salt as a white solid, which was dried under vacuum and used without additional purification (3.95 g, 100%). The HCl salt was then suspended in DCM (40 mL) and THF (50 mL), and tetrahydro-2H-pyran-4-carbaldehyde (2.29 g, 20.1 mmol, 1.4 eq) was added, followed by sodium triacetoxyborohydride (6.08 g, 28.9 mmol, 2 eq). The resulting solution was stirred at r.t. for 1.5 h, after which time the reaction mixture was quenched with sat. $NaHCO_3$, and extracted with DCM. Combined organic extracts were washed with brine, and dried over $MgSO_4$. Solvents were filtered and concentrated to give the title compound as a white solid (4.31 g, 89% over 2 steps).

^{1}H-NMR (400 MHz, MeOD) δ 7.26 (d, *J* = 9.4 Hz, 1H), 6.86 (d, *J* = 9.4 Hz, 1H), 4.43 – 4.36 (m, 1H), 3.93 (dd, *J* = 11.3, 3.7 Hz, 2H), 3.42 (td, *J* = 11.9, 1.9 Hz, 2H), 2.84 – 2.68 (m, 4H), 2.31 (d, *J* = 6.8 Hz, 2H), 2.27 – 2.17 (m, 2H), 1.91 (ddd, *J* = 12.9, 5.9, 2.1 Hz, 2H), 1.83 – 1.64 (m, 5H), 1.32 – 1.21 (m, 2H).

^{13}C-NMR (101 MHz, MeOD) δ 159.7, 146.6, 130.3, 120.4, 68.9, 63.5, 62.9, 53.4, 41.5, 39.1, 35.3, 32.9.

ES-MS $[M+H]^+$ = 337.2.

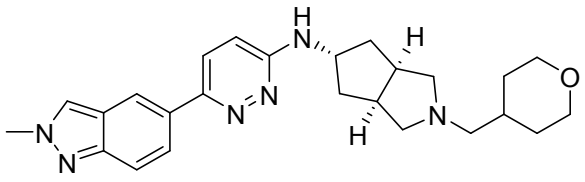


(3a*R*,5s,6a*S*)-*N*-(6-(2-chloro-5-fluorophenyl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (6, VU6013720) (3a*R*,5s,6a*S*)-*N*-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (**5**) (100 mg, 0.30 mmol, 1 eq), 2-chloro-5-fluorophenylboronic acid (62 mg, 0.36 mmol, 1.2 eq), potassium carbonate (125 mg, 0.89 mmol, 3 eq) and BrettPhos-Pd-G3 (27 mg, 0.030 mmol, 0.1 eq) were combined in a vial, and 5:1 1,4-dioxane/H_2O solution (5 mL total, degassed under vacuum) was added via syringe. The resulting mixture was stirred under an inert atmosphere at 100 °C for 2.5 h, after which time the reaction mixture was cooled to r.t. and diluted with water and DCM. The aqueous layer was extracted with DCM, and combined organic extracts were filtered through a phase separator and concentrated. Crude residue was purified by RP-HPLC (10-50% MeCN in 0.1% TFA aqueous solution over 20 min). Fractions containing product were basified with sat. $NaHCO_3$, and extracted with DCM. Combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure to give the title compound as a white solid (40 mg, 31%).

^{1}H-NMR (400 MHz, MeOD) δ 7.57 – 7.52 (m, 2H), 7.35 (dd, *J* = 9.0, 3.1 Hz, 1H), 7.20 (ddd, *J* = 8.8, 7.9, 3.1 Hz, 1H), 6.91 (d, *J* = 9.3 Hz, 1H), 4.56 – 4.48 (m, 1H), 3.94 (dd, *J* = 11.2, 3.5 Hz, 2H), 3.43 (td, *J* = 11.9, 1.9 Hz, 2H), 2.87 – 2.74 (m, 4H), 2.34 (d, *J* = 6.9 Hz, 2H), 2.26 (dd, *J* = 8.4, 4.0 Hz, 2H), 1.97 (ddd, *J* = 12.9, 5.9, 2.1 Hz, 2H), 1.84 – 1.70 (m, 5H), 1.34 – 1.22 (m, 2H).

^{13}C-NMR (101 MHz, MeOD) δ 162.8 (d, *J* = 246.1 Hz), 159.7, 150.95 (d, *J* = 1.9 Hz), 139.8 (d, *J* = 8.1 Hz), 132.8 (d, *J* = 8.5 Hz), 130.5, 128.6 (d, *J* = 3.3 Hz), 118.9 (d, *J* = 24.1 Hz), 118.0 (d, *J* = 23.0 Hz), 116.2, 68.9, 63.6, 63.0, 53.3, 41.5, 39.3, 35.3, 33.0.

ES-MS $[M+H]^+$ = 431.4.

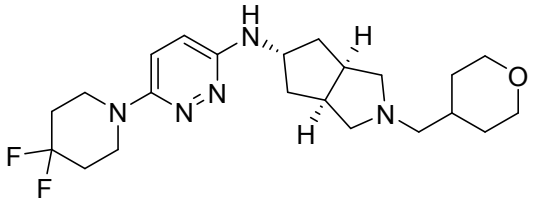


(3a*R*,5s,6a*S*)-*N*-(6-(2-methyl-2H-indazol-5-yl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (7, VU6021625). (3a*R*,5s,6a*S*)-*N*-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (**5**) (1.0 g, 2.97 mmol, 1 eq), 2-methylindazole-5-boronic acid pinacol ester (996 mg, 3.86 mmol, 1.3 eq), potassium carbonate (1.25 g, 8.91 mmol, 3 eq) and BrettPhos-Pd-G3 (269 mg, 0.30 mmol, 0.1 eq) were combined in a vial, and 5:1 1,4-dioxane/H_2O solution (15 mL total, degassed under vacuum) was added via syringe. The resulting mixture was stirred under an inert atmosphere at 100 °C for 3 h, after which time the reaction mixture was cooled to r.t. and diluted with water and DCM. The aqueous layer was extracted with DCM, and combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Crude residue was purified by RP-HPLC (20-60% MeCN in 0.05% NH_4OH aqueous solution over 20 min). Fractions containing product were concentrated to give the title compound as a white solid (431 mg, 34%).

^{1}H-NMR (400 MHz, MeOD) δ 8.27 (s, 1H), 8.17 (dd, *J* = 1.7, 0.9 Hz, 1H), 7.95 (dd, *J* = 9.1, 1.7 Hz, 1H), 7.78 (d, *J* = 9.4 Hz, 1H), 7.67 (dt, *J* = 9.1, 1.0 Hz, 1H), 6.93 (d, *J* = 9.4 Hz, 1H), 4.54 – 4.47 (m, 1H), 4.23 (s, 3H), 3.94 (dd, *J* = 11.0, 3.4 Hz, 2H), 3.43 (td, *J* = 11.9, 2.0 Hz, 2H), 2.91 – 2.73 (m, 4H), 2.36 (d, *J* = 6.9 Hz, 2H), 2.30 – 2.26 (m, 2H), 2.00 – 1.95 (m, 2H), 1.85 – 1.70 (m, 5H), 1.33 – 1.22 (m, 2H).

^{13}C-NMR (101 MHz, MeOD) δ 159.3, 152.5, 150.1, 131.9, 127.4, 127.3, 126.3, 123.6, 119.0, 117.8, 68.9, 63.5, 62.9, 53.3, 41.5, 40.3, 39.2, 35.2, 32.9. *Note: 1 aromatic signal is obscured.*

ES-MS $[M+H]^+$ = 433.0.



(3a*R*,5s,6a*S*)-*N*-(6-(4,4-difluoropiperidin-1-yl)pyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (8, VU6021302). (3a*R*,5s,6a*S*)-*N*-(6-chloropyridazin-3-yl)-2-((tetrahydro-2H-pyran-4-yl)methyl)octahydrocyclopenta[c]pyrrol-5-amine (**5**) (1.0 g, 2.96 mmol, 1 eq) and 4,4-difluoropiperidine hydrochloride (4.68 g, 29.7 mmol, 10 eq) were combined in NMP (10 mL), and DIPEA (5.17 mL, 29.7 mmol, 10 eq) was added. The resulting solution was stirred under microwave irradiation at 200 °C for 2 h, after which time the reaction mixture was purified directly by RP-HPLC (25-65% MeCN in 0.05% NH_4OH aqueous solution over 20 min). Fractions containing product were concentrated to give the title compound as a slightly tan solid (797 mg, 64%).

^{1}H-NMR (400 MHz, MeOD) δ 7.18 (d, *J* = 9.7 Hz, 1H), 6.81 (d, *J* = 9.7 Hz, 1H), 4.36 – 4.29 (m, 1H), 3.93 (dd, *J* = 11.1, 3.4 Hz, 2H), 3.57 – 3.54 (m, 4H), 3.42 (td, *J* = 11.8, 2.0 Hz, 2H), 2.88 – 2.86 (m, 2H), 2.79 – 2.69 (m, 2H), 2.34 (d, *J* = 6.9 Hz, 2H), 2.23 (dd, *J* = 9.3, 5.1 Hz, 2H), 2.08 – 1.98 (m, 4H), 1.91 (ddd, *J* = 12.9, 6.1, 2.3 Hz, 2H), 1.83 – 1.62 (m, 5H), 1.31 – 1.21 (m, 2H).

^{13}C-NMR (101 MHz, MeOD) δ 155.9, 155.7, 123.3 (t, J = 240.8 Hz), 120.8, 120.7, 68.9, 63.5, 62.9, 53.4, 45.2 (t, J = 5.2 Hz), 41.4, 39.2, 35.2, 34.3 (t, J = 23.0 Hz), 32.9.

ES-MS $[M+H]^+$ = 422.5.

Scheme S1. Chemistry Supporting Information. Additional synthesis, ^{1}H-NMR, and ^{13}C-NMR details for intermediates and final compounds

	[^{3}H] NMS Kd (nM)	Bmax (fmol/mg)
ratM1-CHO	0.088 ± 0.013	1305 ± 208
ratM2/Gqi5-CHO	0.155 ± 0.016	2146 ± 223
ratM3-CHO	0.077 ± 0.007	1126 ± 123
ratM4/Gqi5-CHO	0.067 ± 0.022	2178 ± 731
ratM5-CHO	0.235 ± 0.041	1701 ± 258
hM1-CHO	0.075 ± 0.004	1479 ± 129
hM2/Gqi5-CHO	0.114 ± 0.012	2089 ± 561
hM3-CHO	0.116 ± 0.014	2233 ± 737
hM4/Gqi5-CHO	0.041± 0.004	703 ± 103
hM5-CHO	0.376 ± 0.088	2633 ± 97

Table S1. Radioligand binding data for stable cell lines. Kd and Bmax values for cell lines stably expressing rat or human muscarinic receptor subtypes. Values are from at least 3 replicates.

Target	Radioligand	Species	% Inhibition
Adenosine A1	[3H] DPCPX	Human	12
Adenosine A2A	[3H] CGS-21680	Human	5
Adenosine A3	[125I] AB-MECA	Human	10
Adrenergic a1A	[3H] Prazosin	Rat	13
Adrenergic a1B	[3H] Prazosin	Rat	3
Adrenergic a1D	[3H] Prazosin	Human	17
Adrenergic a2A	[3H] Rauwolscine	Human	18
Adrenergic ß1	[125I] Cyanopindolol	Human	6
Adrenergic ß2	[3H] CGP-12177	Human	0
Androgen (Testosterone)	[3H] Methyltrienolone	Human	-13
Bradykinin B1	[3H] (Des-Arg10, Leu9)- Kallidin	Human	-10
Bradykinin B2	[3H] Bradykinin	Human	-7
Calcium Channel L-Type, Benzothiazepine	[3H] Diltiazem	Human	7
Calcium Channel L-Type, Dihydropyridine	[3H] Nitrendipine	Rat	1
Calcium Channel N-Type	[125I] ω-Conotoxin GVIA	Rat	-9
Cannabinoid CB1	[3H] SR141716A	Rat	-3
Dopamine D1	[3H] SCH-23390	Human	2
Dopamine D2S	[3H] Spiperone	Human	17
Dopamine D3	[3H] Spiperone	Human	34
Dopamine D4.4	[3H] Spiperone	Human	3

Endothelin ETA	[125I] Endothelin-1	Human	-11
Endothelin ETB	[125I] Endothelin-1	Human	2
Epidermal Growth Factor (EGF)	[125I] EGF	Human	-5
Estrogen ERa	[3H] Estradiol	Human	-3
GABAA, Flunitrazepam, Central	[3H] Flunitrazepam	Rat	-4
GABAA, Muscimol, Central	[3H] Muscimol	Rat	-11
GABAB1A	[3H] CGP-54626	Human	2
Glucocorticoid	[3H] Dexamethasone	Human	-2
Glutamate, Kainate	[3H] Kainic acid	Rat	6
Glutamate, NMDA, Agonism	[3H] CGP-39653	Rat	-5
Glutamate, NMDA, Glycine	[3H] MDL 105,519	Rat	-12
Glutamate, NMDA, Phencyclidine	[3H] TCP	Rat	-1
Histamine H1	[3H] Pyrilamine	Human	25
Histamine H2	[125I] Aminopotentidine	Human	-3
Histamine H3	[3H] N-α-Methylhistamine	Human	88
Imidazoline I2, Central	[3H] Idazoxan	Rat	2
Interleukin IL-1 R1	[125I] Interleukin-1β	Human	-7
Leukotriene, Cysteinyl CysLT1	[3H] LTD4	Human	12

Melatonin MT1	[125I] 2-Iodomelatonin	Human	-2
Muscarinic M1	[3H] N-Methylscopolamine	Human	33
Muscarinic M2	[3H] N-Methylscopolamine	Human	85
Muscarinic M3	[3H] N-Methylscopolamine	Human	51
Neuropeptide Y Y1	[125I] Peptide YY	Human	-13
Neuropeptide Y Y2	[125I] Peptide YY	Human	-2
Nicotinic Acetylcholine α1, Bungarotoxin	[125I] α-Bungarotoxin	Human	-1
Nicotinic Acetylcholine α3β4	[125I] Epibatidine	Human	55
Opiate δ1 (OP1, DOP)	[3H] Naltrindole	Human	-1
Opiate κ (OP2, KOP)	[3H] Diprenorphine	Human	5
Opiate μ (OP3, MOP)	[3H] Diprenorphine	Human	6
Phorbol Ester	[3H] PDBu	Mouse	9
Platelet Activating Factor (PAF)	[3H] PAF	Human	-4
Potassium Channel [KATP]	[3H] Glyburide	Human	11
Potassium Channel hERG	[3H] Astemizole	Human	23
Prostanoid EP4	[3H] Prostaglandin E2	Human	0
Purinergic P2X	[3H] α, β-Methylene-ATP	Rat	4
Rolipram	[3H] Rolipram	Rat	0

Serotonin (5-Hydroxytryptamine) 5-HT1A	[3H] 8-OH-DPAT	Human	6
Serotonin (5-Hydroxytryptamine) 5-HT2B	[3H] Lysergic acid diethylamide	Human	53
Serotonin (5-Hydroxytryptamine) 5-HT3	[3H] GR-65630	Human	-2
Sigma σ1	[3H] Haloperidol	Human	38
Sodium Channel, Site 2	[3H] Batrachotoxinin	Rat	15
Tachykinin NK1	[3H] Substance P	Human	-10
Thyroid Hormone	[125I] Triiodothyronine	Rat	-21
Transporter, Dopamine (DAT)	[125I] RTI-55	Human	15
Transporter, GABA	[3H] GABA	Rat	-5
Transporter, Norepinephrine (NET)	[125I] RTI-55	Human	15
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	[3H] Paroxetine	Human	-1

Table S2. Ancillary Pharmacology of VU6021625. Eurofins panel showing binding of VU6021625 to a screen of 88 different receptors, transporters and enzymes.

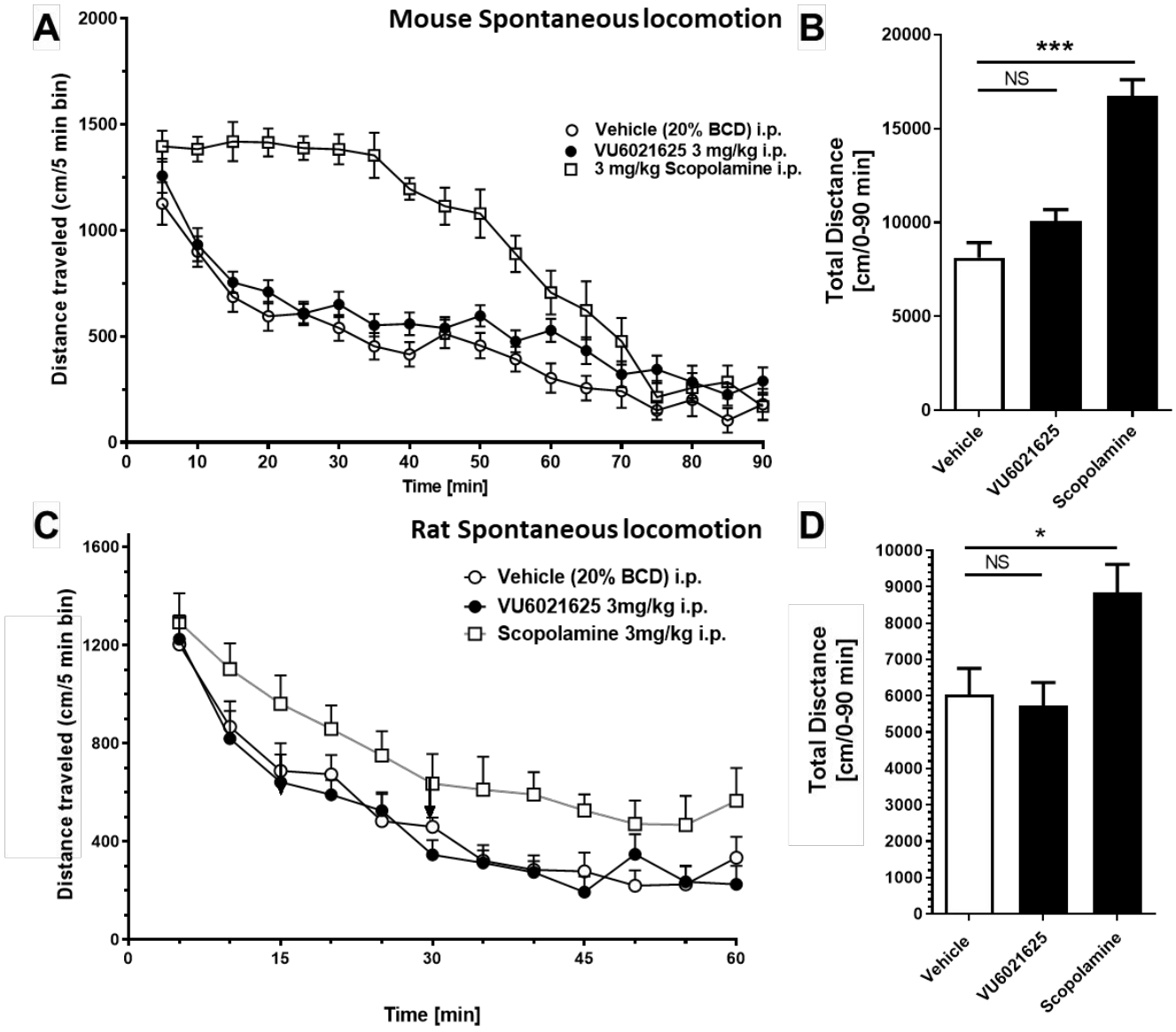


Figure S1. VU6021625 does not alter spontaneous locomotion**.** Unlike scopolamine (3 mg/kg, i.p.), VU6021625 (3 mg/kg, i.p.) does not increase spontaneous locomotor activity in either mice (A-B) or rats (C-D), (A, C time activity curve; B, D total distance travelled after vehicle, VU6021625, or scopolamine administration). Mouse N=12 per group. Rat N=8 per group. One way ANOVA with Dunnett's post-hoc test **p<0.01, ***p<0.001, NS, not significant.

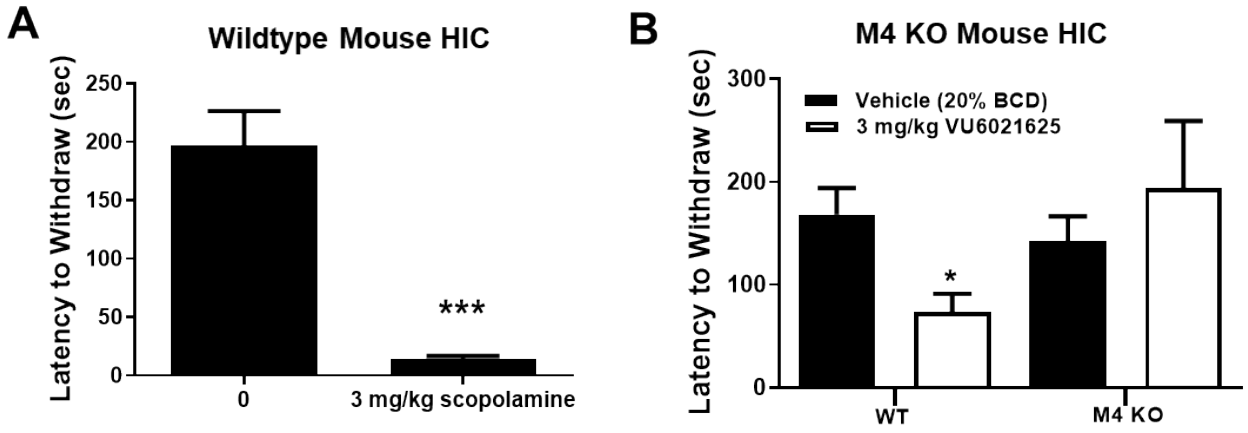


Figure S2. Wildtype and M_4 knockout mouse haloperidol induced catalepsy. Comparison group of a maximally efficacious dose of scopolamine in reversing catalepsy (A). Systemic administration of VU6021625 demonstrates efficacy in reversing catalepsy in wildtype, but not M_4 global knockout animals (B). N=8 per group. Student's t test per. N=4-12 per group. One way ANOVA with Dunnett's post-hoc test **p<0.01, ***p<0.001, NS, not significant