Supplemental Figures and Legends

Figure S1. Dose-response relationship of M_4 PAM VU0467154 reversal of D_1 agonist-induced hyperlocomotion. Related to Figure 1

Figure S2. Localization of M_4 in the striatum and substantia nigra pars reticulata. Related to Figure 2

Figure S3. Origination and density of cholinergic fibers in the substantia nigra pars reticulata. Related to Figure 2

Figure S4. D_1 agonists and muscarinic agents do not alter mIPSC amplitude in GABAergic cells of wildtype or D_1-M_4 knockout mice. Related to Figures 2 and 3

Figure S5. Forskolin mimics the GABA releasing effects of D_1 agonists and is blocked by M4 activation on D_1-SPNs. Related to Figure 5

Figure S6. M_4 activation increases rise time of CBV change in response to D_1 agonists in the cingulate cortex. Related to Figure 6

Figure S7. M_4 potentiation has no detectable CBV fMRI effect in subcortical and cortical regions. Related to Figure 6

Table S1. Non-Normalized mIPSC inter event interval and amplitude values. Related to Figures 2, 3, 4, and 5

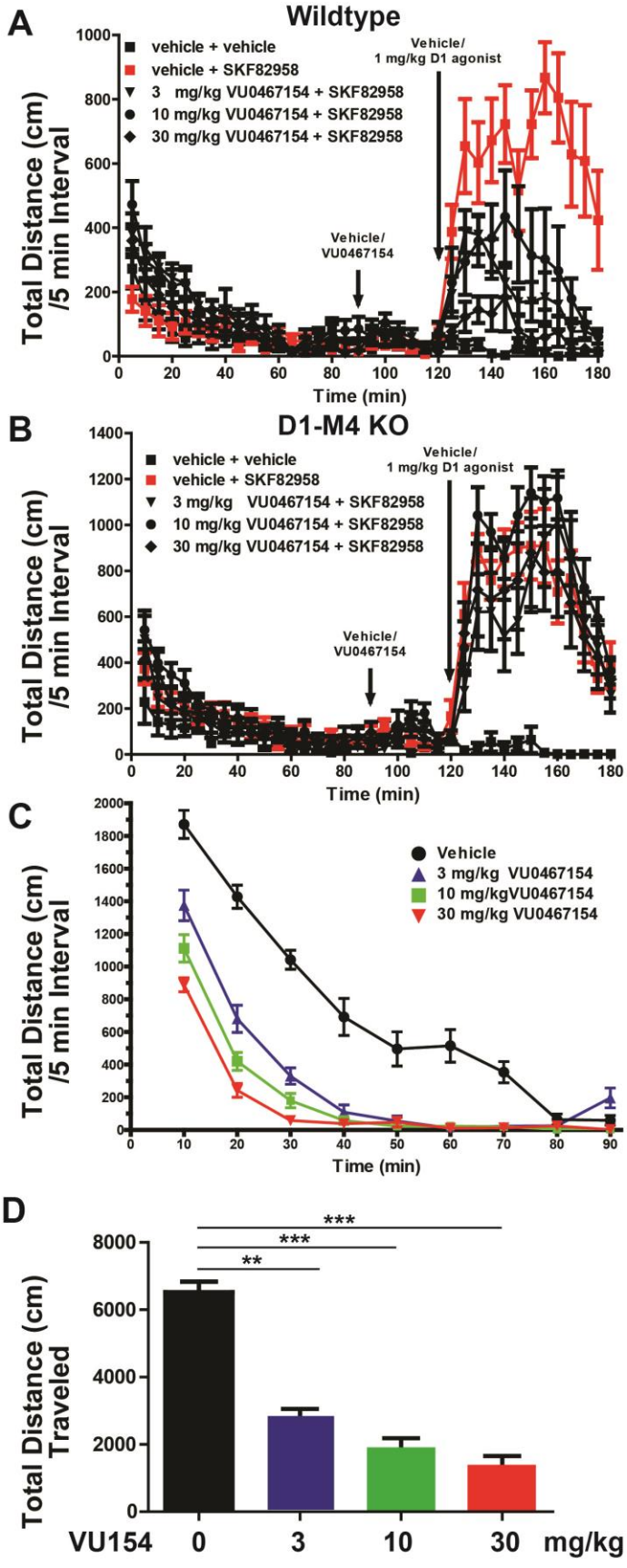

A
Wildtype
vehicle + vehicle
vehicle + SKF82958
3 mg/kg VU0467154 + SKF82958
10 mg/kg VU0467154 + SKF82958
30 mg/kg VU0467154 + SKF82958
Vehicle/
1 mg/kg D1 agonist
Vehicle/
VU0467154
Total Distance (cm)
/5 min Interval
Time (min)
B
D1-M4 KO
vehicle + vehicle
vehicle + SKF82958
3 mg/kg VU0467154 + SKF82958
10 mg/kg VU0467154 + SKF82958
30 mg/kg VU0467154 + SKF82958
Vehicle/
1 mg/kg D1 agonist
Vehicle/
VU0467154
Total Distance (cm)
/5 min Interval
Time (min)
C
Vehicle
3 mg/kg VU0467154
10 mg/kgVU0467154
30 mg/kg VU0467154
Total Distance (cm)
/5 min Interval
Time (min)
D
***
***
**
Total Distance (cm)
Traveled
VU154 0 3 10 30 mg/kg

Figure S1. Related to Figure 1. Dose-response relationship of M_4 PAM VU0467154 reversal of D_1 agonist-induced hyperlocomotion. A) Wildtype (WT) animals were injected with M_4 PAM VU0467154 (3, 10, or 30 mg/kg, intraperitoneal (i.p.), 10% Tween 80) 90 minutes after being placed in locomotion chambers. Thirty minutes later, D1 agonist SKF82958 was administered (1 mg/kg, i.p., sterile water). Activity was then recorded for an additional 60 min and reported as distance in centimeters (cm) per 5 minute bins. **B)** Animals with M_4 selectively knocked out in DRD_1 expressing striatal spiny projection neurons (D_1-M_4 KO) were injected with M_4 PAM VU0467154 (3, 10, or 30 mg/kg, i.p., 10% Tween 80) 90 minutes after being placed in locomotion chambers. Thirty minutes after VU0467254 or vehicle injection, D_1 agonist SKF82958 was administered (1 mg/kg, i.p., sterile water). **C)** Wildtype (WT) animals were injected with M_4 PAM VU0467154 (3, 10, or 30 mg/kg, intraperitoneal (i.p.), 10% Tween 80) 30 minutes before being placed in locomotion chambers. Activity was then recorded for 90 min and reported as distance in centimeters (cm) per 5 minute bins. **D)** Total distance moved in cm from animals in **(A)** after placement in locomotion chambers. Data are mean ± SEM with an n=8-11 per group. ** indicates $p<0.01$, *** indicates $p<0.001$ by one way ANOVA followed by Tukey's post-hoc test.

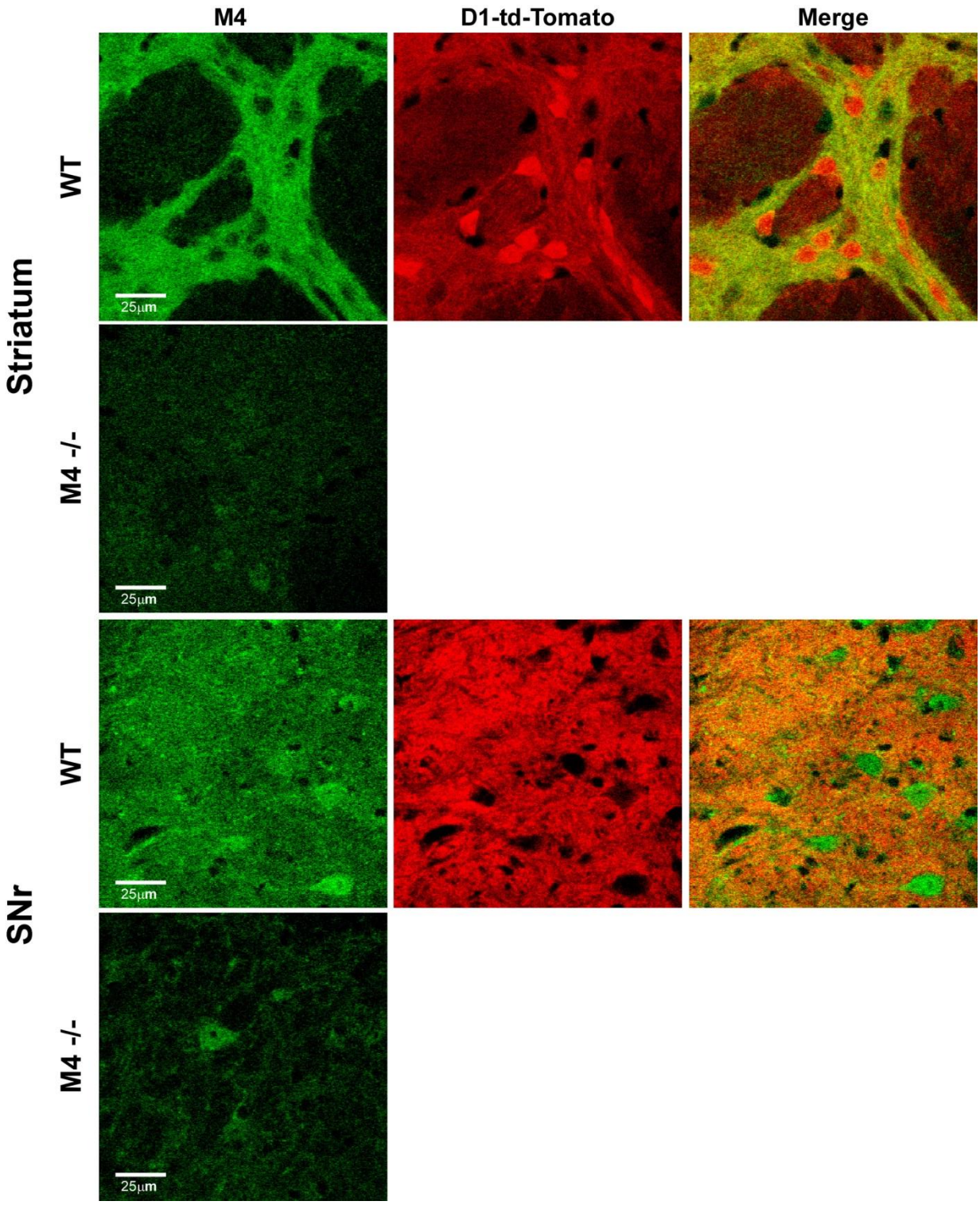


Figure S2. Related to Figure 2. Localization of M_4 in the striatum and substantia nigra pars reticulata. Transgenic animals expressing the fluorescent protein td-Tomato under the transcriptional control of the *DRD_1* promoter were used to visualize cells and projections of the direct pathway and then stained for M_4. In the substantia nigra pars reticulata (SNr), robust

expression (green) co-localizes with td-Tomato positive puncta indicating that M_4 is expressed in DRD_1-expressing striatal spiny projection neuron terminals. The M_4 signal is also seen in SNr cell bodies and this staining is also observed in sections derived from in M_4 global knockout mice (M_4 -/-), suggesting an off target effect of the antibody. In the striatum, a diverse pattern of M_4 expression is seen as previously reported (see Levey et al 1991). M_4 staining can be seen inside of DRD_1-expressing striatal spiny projection neurons as well as in the neuropil.

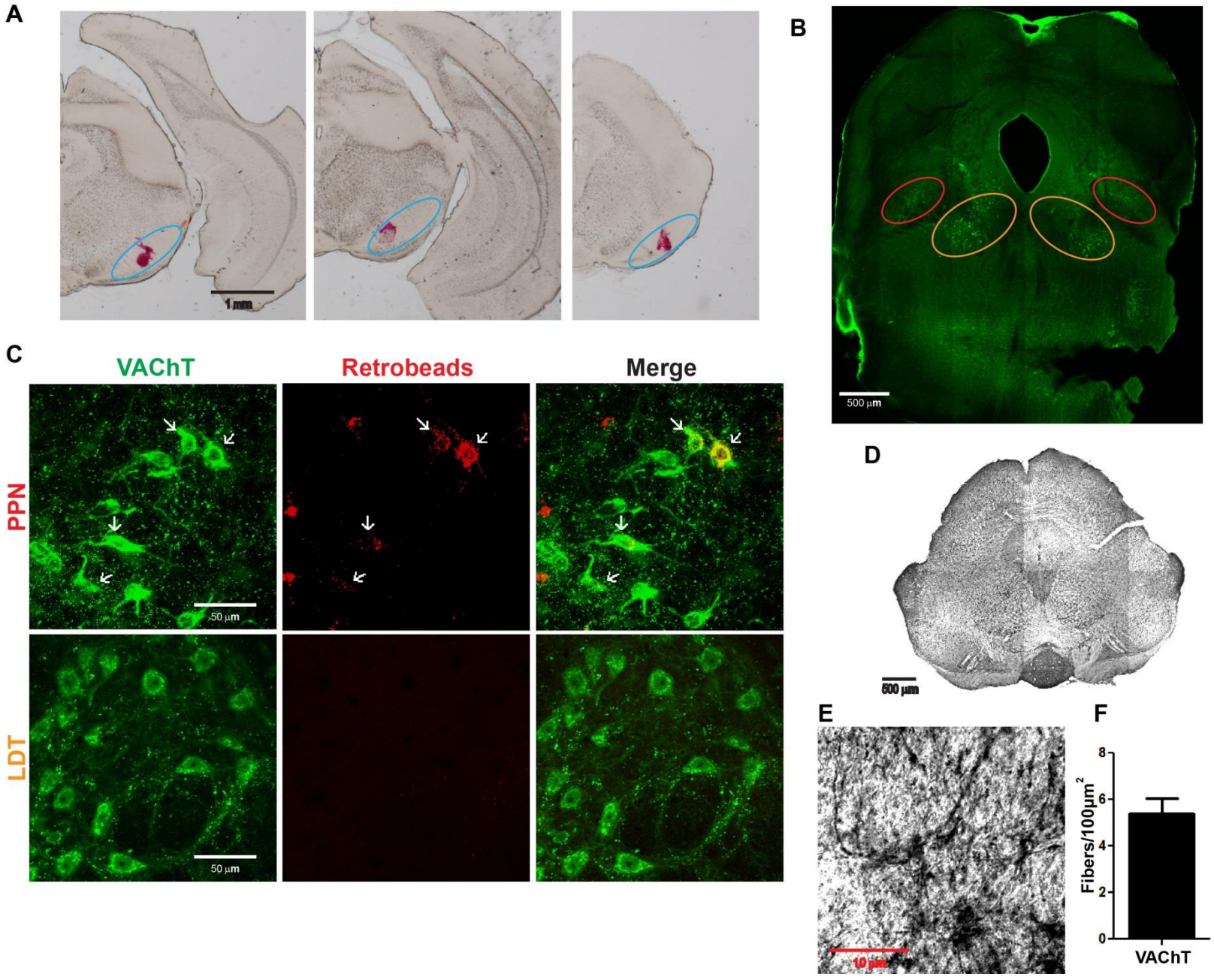

A
1 mm
B
500 µm
C
VAChT
Retrobeads
Merge
PPN
LDT
50 µm
50 µm
D
500 µm
E
10 µm
F
Fibers/100µm²
8
6
4
2
0
VAChT

Figure S3. Related to Figure 2. Origination and density of cholinergic fibers in the substantia nigra pars reticulata. A) Wildtype (WT) animals were injected with 100 nL of red fluorescent retrobeads into the substantia nigra pars reticulata (SNr). Animals were allowed to sit with beads injected for at least one week before sacrifice. Coronal sections were made from post-fixed brains and imaged using a widefield microscope. Blue ellipses indicate the borders of the SNr. The sphere of injection is almost always inside the SNr and only rarely strays outside of the SNr. **B)** The hindbrains of animals from **(A)** were sectioned at 40 μm and stained with antibodies against the vesicular acetylcholine transported (VAChT) and appropriate secondary antibody. Widefield images of hindbrain sections revealed both the PPN (red ellipses) and LDT (orange ellipses). **C)** Zoomed in images (63X objective) of PPN and LDT areas from **(B)**. Several VAChT positive cells could be identified in both the PPN and LDT, but retrobeads were only identified in cells of PPN. **D)** Widefield DAB image of coronal brain section containing the SNr and stained with VAChT. **E)** 100X image of the SNr from **(D)**. **F)** Estimation of the cholinergic fiber density from **(E)** using an ImageJ plugin.

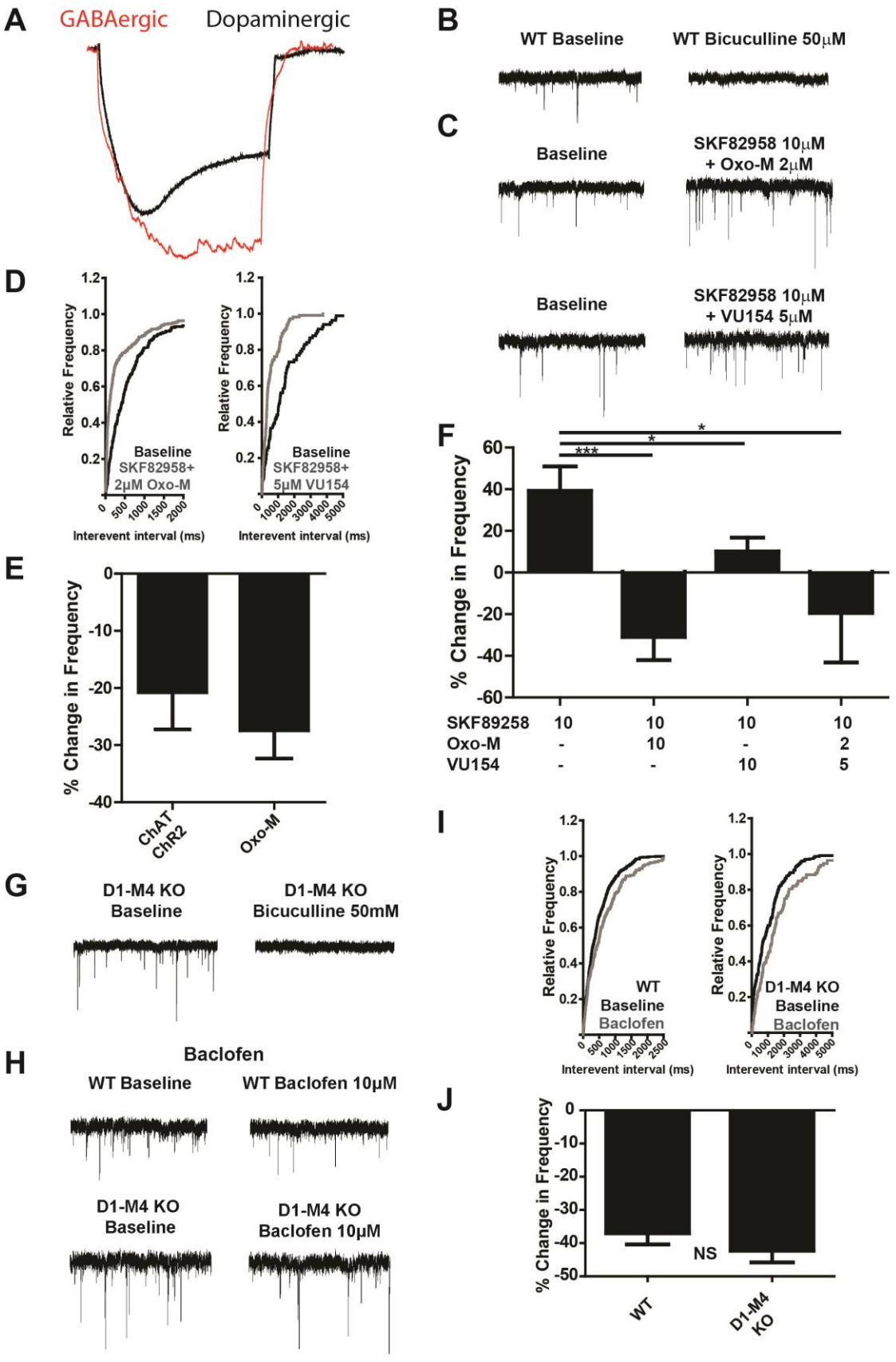

A
GABAergic
Dopaminergic
B
WT Baseline
WT Bicuculline 50μM
C
Baseline
SKF82958 10μM
+ Oxo-M 2μM
Baseline
SKF82958 10μM
+ VU154 5μM
D
Relative Frequency
Baseline
SKF82958+
2μM Oxo-M
Interevent interval (ms)
Baseline
SKF82958+
5μM VU154
Interevent interval (ms)
E
% Change in Frequency
ChAT
ChR2
Oxo-M
F
% Change in Frequency
SKF89258 10 10 10 10
Oxo-M - 10 - 2
VU154 - - 10 5
G
D1-M4 KO
Baseline
D1-M4 KO
Bicuculline 50mM
H
Baclofen
WT Baseline
WT Baclofen 10μM
D1-M4 KO
Baseline
D1-M4 KO
Baclofen 10μM
I
Relative Frequency
WT
Baseline
Baclofen
Interevent interval (ms)
D1-M4 KO
Baseline
Baclofen
Interevent interval (ms)
J
% Change in Frequency
NS
WT
D1-M4
KO

Figure S4. Related to Figure 2. D_1 agonists and muscarinic agents do not alter mIPSC amplitude in GABAergic cells of wildtype or D_1-M_4 knockout mice. A) Representative CCIV plots of GABAergic (red) and dopaminergic (black) cells of the SNr as soon as whole cell was achieved. This, as well as firing rate (GABA >10 Hz and DA 1-5 Hz), revealed SNr cell type. **B)** Representative mIPSC traces from GABAergic cells of the SNr before and after treatment with 50 μM bicuculline, which removed any detectable events. **C)** Representative mIPSC traces from GABAergic cells of the SNr treated with 10 μM SKF82958 and 2 μM Oxotremorine-M (Oxo-M) or 5 μM VU0467154 in WT mice. **D)** Cumulative probability plots of traces in **(C). E)** Optical stimulation of cholinergic afferents in the SNr from ChAT-ChR2 mice reduced mIPSC frequency by ~20% at baseline which was similar to the effect of application on 10μM Oxo-M alone. This indicates that activation of cholinergic afferents alone in the SNr is sufficient to regulate D_1-SPN terminals. **F)** Summary of data from slices warmed to 30C before patching and treating with the drugs indicated. Data at 30C recapitulates our findings from Figure 2. **G)** Representative mIPSC traces from GABAergic cells of the SNr before and after treatment with 50 μM bicuculline, which removed any detectable events. **H)** Representative mIPSC traces from GABAergic cells of the SNr treated with 10 μM baclofen from wildtype (WT) or D1-M4 KO mice. **I)** Cumulative probability plots of traces in **(H). J)**Summary of data represented in **(H, I)**. Negative modulations correspond to decreased mIPSC frequency as compared to baseline. Positive modulation corresponds to an increased mIPSC frequency and negative modulation corresponds to decreased mIPSC frequency as compared to baseline. Data are mean ± SEM with an n=8-10 per group. * indicates $p<0.05$, *** indicates $p<0.001$ by Kruksal-Wallis test followed by Dunnett's post-test. In **(I)** NS indicates not statistically significant by two-tailed t-test.

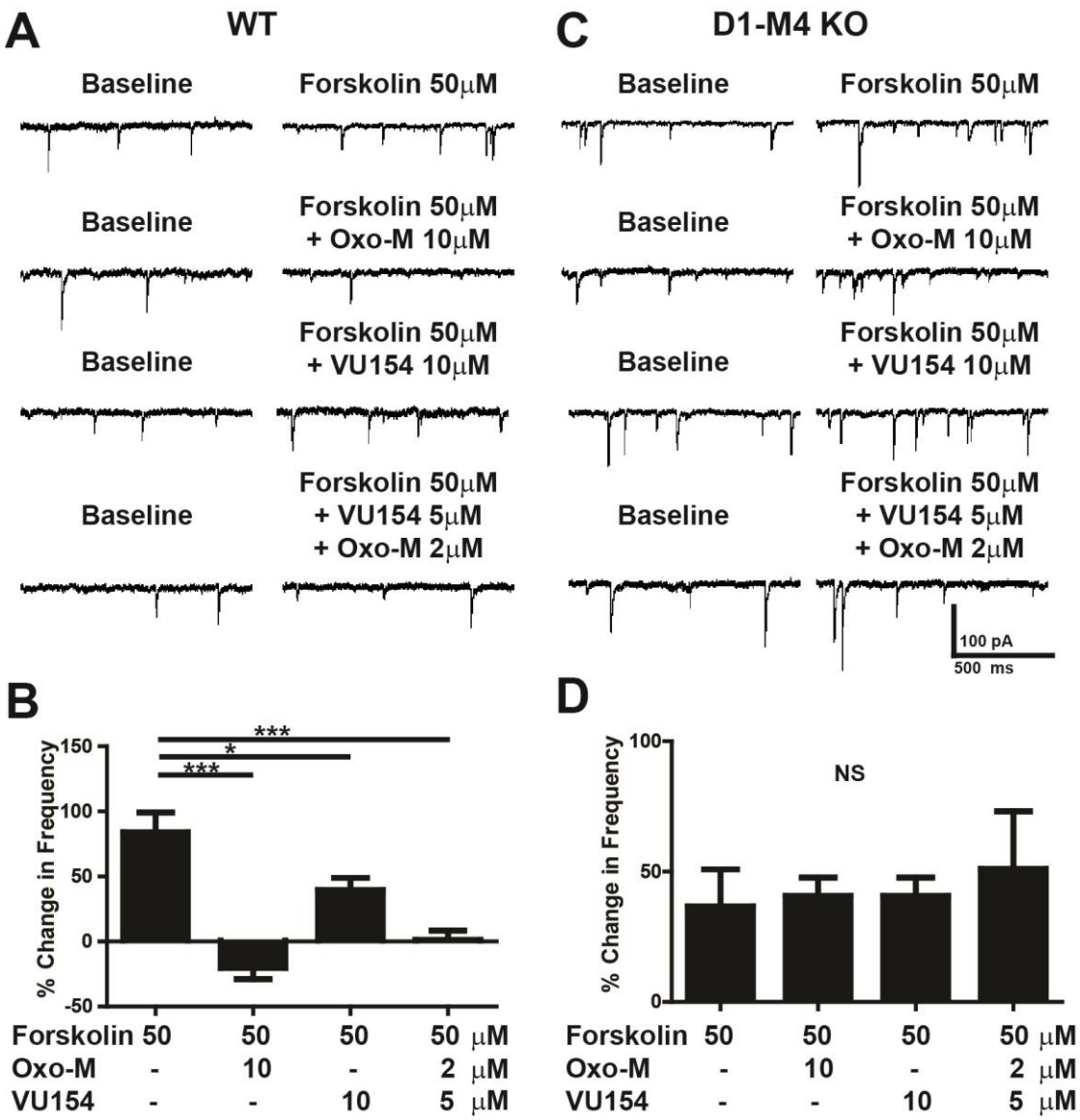


Figure S6. Related to Figure 5. Forskolin mimics the GABA releasing effects of D_1 agonists and is blocked by M_4 activation on D_1-SPNs. A) Representative miniature inhibitory post synaptic currents (mIPSC) traces from GABAergic cells of the substantia nigra pars reticulata (SNr) treated with 50 μM forskolin and 10 μM Oxotremorine-M (Oxo-M), 10 μM VU04567154, or 2 μM Oxo-M and 5 μM VU0467154 in WT mice **B)** Cumulative probability plots of traces in **(A)**. **C)** Summary of data represented in **(A, B)** Positive modulation corresponds to an increased mIPSC frequency and negative modulation corresponds to decreased mIPSC frequency as compared to baseline. **D)** Representative mIPSC traces from GABAergic cells of the SNr treated with 50 μM forskolin and 10 μM Oxotremorine-M (Oxo-M), 10 μM VU04567154, or 2 μM Oxo-M and 5 μM VU0467154 in mice with M_4 selectively knocked out in DRD_1-expressing striatal spiny projection neurons (D_1-M_4 KO). **E)** Cumulative probability plots of traces in **(D). F)** Graph of data represented in **(D). E)** Positive modulation corresponds to an increased mIPSC frequency and negative modulation corresponds to decreased mIPSC

frequency as compared to baseline. Data are mean ± SEM with an n=8-12 per group. ** indicates $p<0.01$, *** indicates $p<0.001$, NS indicates non-significant by one way ANOVA followed by Tukey's post-hoc test.

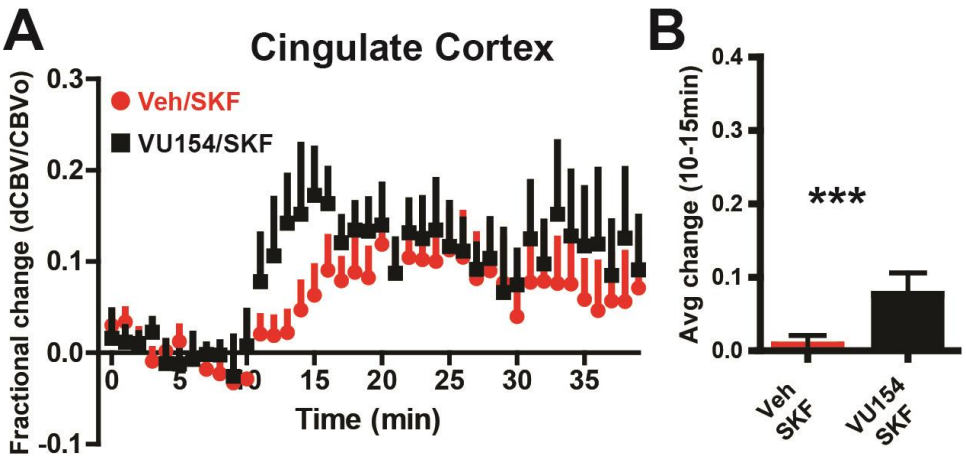
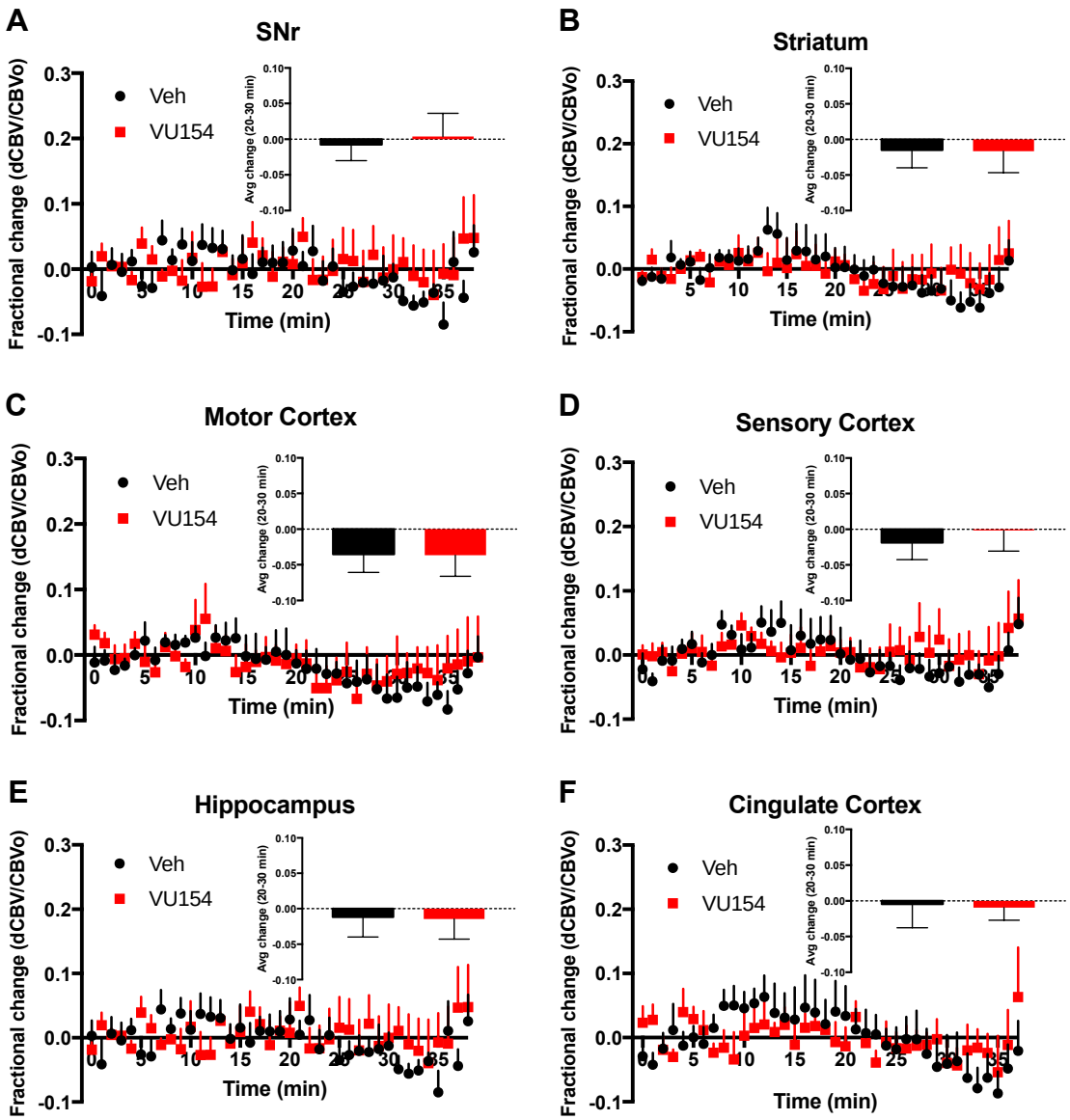


Figure S7. Related to Figure 6. M_4 activation increases rise time of CBV change in response to D_1 agonists in the cingulate cortex. A) Timeline of cerebral blood volume (CBV) changes after D_1 agonist SKF82958 injection (1mg/kg, intraperitoneal (i.p.), sterile water) in male Sprague-Daley rats pretreated with vehicle (i.p., 10% Tween 80) or 30 mg/kg M_4 PAM VU0467154 (i.p., 10% Tween 80) from the cingulate cortex. Timeline graph shows fractional changes in CBV which are calculated by dividing the CBV signal for each region at each time point (dCBV) by the CBV for each region collected during the baseline period (CBVo) before SKF82958 injection. Positive numbers indicate increased CBV, correlating with increased neuronal activity, while negative numbers indicate decreased CBV, correlating with decreased neuronal activity, compared to baseline. **B)** Graph of data from **(A)**, fractional CBV values were averaged between 10 to 15 minutes to isolate the rising portion of the graph. Data are mean ± SEM with an n=6 per group. *** indicates $p<0.001$ by Mann-Whitney test.



Supplemental Figure S8. Related to Figure 6. M_4 potentiation has no detectable CBV fMRI effect in subcortical and cortical regions. A-F) Time courses and bar graphs (insets) of fractional CBV changes after M_4 PAM VU0467154 (30 mg/kg, 10% Tween-80 IP) or vehicle injection in rats in the indicated brain regions. Bar graphs indicate fractional CBV values averaged over time points 20 to 30 minutes. There were no significant effects in any region as indicated by Mann-Whitney U test ($p>0.05$).

Figure 2	Inter-event interval (ms)		Significance	Amplitude (pA)		Significance
WT Mice	Baseline	After Drug		Baseline	After Drug	
SKF82958 10µM	531.73 ± 600.24	358.44 ± 318.36	p<0.05	71.1 ± 13.2	81.9 ± 22.5	NS
+ Oxo-M 2µM	725.4 ± 517.5	560.4 ± 400.9	p<0.05	67.7 ± 31.5	65.2 ± 20.1	NS
+ VU154 5µM	805.2 ± 409.7	645.2 ± 381.4	p<0.05	62.1 ± 13.3	56.5 ± 14.3	NS
+ Oxo-M 10µM	665.923 ± 514.91	818.12 ± 790.84	NS	82.9 ± 11.9	77.7 ± 7.6	NS
+ VU154 10µM	707.9 ± 506.1	735.1 ± 612.4	NS	81.4 ± 20.4	95.1 ± 10.9	NS
+ Oxo-M 2µM +VU154 5µM	708.5 ± 477.5	824.1 ± 479.3	p<0.05	53.1 ± 7.8	50.4 ± 4.6	NS
ChAT ChR2 Mice						
SKF82958 5µM	454.8 ± 321.8	364.1 ± 260.2	p<0.05	87.2 ± 27.3	89.8 ± 24.8	NS
+ VU154 5µM	376 ± 137.6	288.3 ± 153.8	p<0.05	79.2 ± 34.2	77.1 ± 27.5	NS
+ Light	460.1 ± 68.41	352.4 ± 151.9	p<0.05	87.8 ± 31.3	82.1 ± 32.9	NS
+ VU154 5 µM + Light	738.9 ± 510.4	826.4 ± 616.3	NS	80.7 ± 15.2	77.1 ± 23.1	NS
Figure 3						
D1-M4 KO Mice	Baseline	After Drug		Baseline	After Drug	
SKF82958 10µM	315.93 ± 359	266.56 ± 334	p<0.05	93.1 ± 13.9	82.3 ± 26.5	NS
+ Oxo-M 10µM	387.1 ± 230.4	283.2 ± 121.2	p<0.05	80.5 ± 5.0	84.7 ± 15.9	NS
+ VU154 10µM	337.5 ± 62.7	274.8 ± 69.1	p<0.05	93.1 ± 11.8	89.8 ± 18.6	NS
+ Oxo-M 2µM+VU154 5µM	456.0 ± 147.4	321.9 ± 104.2	p<0.05	89.9 ± 15.9	101.6 ± 18.5	NS
CNO Alone	524.9 ± 360.9	659.4 ± 486.0	NS	91.1 ± 16.9	89.8 ± 17.39	NS
SKF82958 10µM + CNO 10µM	724.9 ± 382.9	770.6 ± 405.9	NS	86.6 ± 19.7	95.8 ± 18.1	NS
Figure 4						
WT Mice	Baseline	After Drug		Baseline	After Drug	
Scopolamine	797.5 ± 391.1	501.1 ± 325.7	p<0.01	55.8 ± 7.9	58.4 ± 5.2	NS
MT-3	635.1 ± 502.4	463.5 ± 442.0	p<0.01	66.2 ± 6.0	63.4 ± 10.2	NS
VU154	405.5 ± 328.3	455.8 ± 345.5	p<0.05	59.7 ± 16.7	59.1 ± 17.9	NS
D1-M4 KO Mice						
Scopolamine	369.4 ± 354.2	328.1 ± 314.0	p<0.05	70.9 ± 14.2	75.9 ± 13.6	NS
MT-3	664.9 ± 335.9	629.3 ± 317.4	NS	54.9 ± 33.1	51.1 ± 27.4	NS
VU154	383.8 ± 125.7	396.6 ± 122.1	NS	85.3 ± 32.8	83.6 ± 31.2	NS
ChAT-Cre Mice						
Control Virus	659.3 ± 502.5	430.4 ± 245.8	p<0.01	77.0 ± 4.4	77.5 ± 17.4	NS
Caspase 3 Virus	533.3 ± 488.8	580.5 ± 489.3	NS	76.4 ± 21.5	78.5 ± 25.9	NS
Figure 5						
WT Mice	Baseline	After Drug		Baseline	After Drug	
β2-ChR	664.9 ± 579.7	474.2 ± 392.4	p<0.001	86.8 ± 13.7	82.3 ± 16.8	NS
+ Oxo-M 10µM	498.7 ± 404.7	488.5 ± 388.1	NS	80.9 ± 17.3	86.0 ± 23.2	NS
+ Oxo-M 2µM+VU154 5µM	607.8 ± 704.3	657.7 ± 766.0	NS	82.3 ± 15.8	83.8 ± 16.0	NS
GFP	433.6 ± 249.7	430.7 ± 230.5	NS	62.8 ± 15.9	54.8 ± 10.8	NS
D1-M4 KO Mice						
β2-ChR	552.6 ± 279.0	361.8 ± 212.3	p<0.001	91.6 ± 27.2	96.3 ± 36.2	NS
+ Oxo-M 10µM	419.4 ± 243.2	318.9 ± 218.6	p<0.01	79.2 ±34.2	75.4 ± 30.0	NS
+ Oxo-M 2µM+VU154 5µM	300.2 ± 173.1	236.7 ± 262.0	p<0.05	96.2 ± 17.5	94.2 ± 10.5	NS
GFP	294.9 ± 221.8	277.8 ± 208.8	NS	65.4 ± 15.8	66.5 ± 21.2	NS