Cell Reports

Supplemental Information

Identification of serotonergic neuronal modules that affect aggressive behavior

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Table S1. Aggressive behaviors following *en masse* serotonin neuron silencing

aggressive behaviors	attack bites (#)	threats (#)	pursuits (#)	tail rattle (s)	latency (s)
ePet::cre, RC::Ptox (n=10)	**17.0 ± 1.7***	**30.1 ± 3.4***	1.7 ± 0.5	**3.5 ± 1.0***	56.2 ± 14.4
control siblings (n=20)	8.1 ± 1.7	15.6 ± 3.6	0.8 ± 0.3	1.4 ± 0.4	135.5 ± 29.5

Table S1. Aggressive behaviors following *en masse* serotonin neuron silencing, Related to Figure 1
Measures of aggressive behavior observed in *ePet::cre, RC::Ptox* mice and control siblings demonstrate increased attack bites (M-W U = 38, p = 0.005), lateral threats (M-W U = 51.5, p=0.03), and tail rattling (M-W U = 45, p=0.01) when 5-HT neurons are silenced *en masse*.

Table S2. Aggressive behaviors following intersectionally defined neuron silencing

aggressive behaviors	attack bites (#)	threats (#)	pursuits (#)	tail rattle (s)	latency (s)
Drd1a::cre,Pet1::Flpe, RC::PFtox (n=11)	14.3 ± 2.7*	26.6 ± 4.8*	0.8 ± 0.3	1.1 ± 0.4	90.8 ± 25.5*
control siblings (n=26)	7.3 ± 1.4	13.8 ± 2.9	0.7 ± 0.3	0.7 ± 0.2	171.9 ± 22.9
Drd2::cre, Pet1::Flpe, RC::PFtox (n=11)	17.7 ± 3.0*	34.5 ± 6.3*	2.3 ± 0.8	0.7 ± 0.2	54.5 ± 25.8
control siblings (n=15)	9.2 ±2.2	15.0 ± 3.5	0.8 ± 0.4	1.0 ± 0.4	115.6 ± 31.0

Table S2. Aggressive behaviors following intersectionally defined neuron silencing, Related to Figure 2
Average measures for all recorded aggressive behaviors are shown for *Drd1a/Pet1*- and *Drd2/Pet1*-silenced mice. In addition to elevated numbers of bites, both silenced genotypes displayed more lateral threats (*Drd1a/Pet1*, M-W U=76.5, p=0.025 and *Drd2/Pet1*, M-W U=36, p=0.017) than respective control siblings. Although both genotypes appeared to attack with shorter latencies the differences between silenced and control animals was only significant for *Drd1a/Pet1*-silenced mice (M-W U=82.5, p=0.046).

Table S3. Nonaggressive behaviors measured during resident intruder tests

Non-aggressive behaviors	Control siblings (n=26)	*Drd1a::cre, Pet1::Flpe, RC::PFtox* (n=11)	Non-aggressive behaviors	Control siblings (n=18)	*Drd2::cre, Pet1::Flpe, RC::PFtox* (n=12)
Walking (s)	91.8 ± 3.4	110.7 ± 6.3**	Walking (s)	88.6 ± 4.2	97.2 ± 4.5
Rearing (s)	24.8 ± 2.1	26.6 ± 4.1	Rearing (s)	22.6 ± 2.1	22.4 ± 3.6
Digging (s)	10.2 ± 2.1	5.8 ± 1.5	Digging (s)	8.0 ± 2.1	8.3 ± 2.3
Grooming (s)	7.3 ± 0.6	6.2 ± 1.4	Grooming (s)	7.4 ± 0.7	6.5 ± 1.1
Contact (s)	7.2 ± 1.0	2.5 ± 0.8**	Contact (s)	6.6 ± 1.3	4.9 ± 1.4

Table S3. Nonaggressive behaviors measured during resident intruder tests, Related to Figure 2
Several non-aggressive behaviors were tracked during the resident intruder tests. *Drd1a/Pet1*-silenced mice spent more time walking around the test chamber (M-W U=67, p=0.016), but spent less time in contact with intruders as compared to controls (M-W U=48, p=0.002). No differences were observed in rearing, digging, or grooming. No differences in any of these behaviors were observed in *Drd2/Pet1*-silenced mice.

Table S4: Statistics for behavioral phenotyping of *Drd1a/Pet1*-silenced mice

Behavior Test	Measurement	Statistical Test	Comparison	F	df	p	Fig.
open field	ambulatory distance	repeated measures ANOVA	F1- genotype	F=3.063	1, 22	0.094	3A
			F2- time	**F=48.294**	**11, 242**	**<0.001*** **	
			(F1xF2)	F=0.419	11, 242	0.947	
	total amb. distance	unpaired t-test	con vs TG	t=-1.750	1, 22	0.094	3B
	vertical time	repeated measures ANOVA	F1- genotypes	F=1,282	1, 22	0.27	3C
			F2- time	**F=3.044**	**11, 242**	**<0.001*** **	
			(F1xF2)	**F=2.022**	**11, 242**	**0.027***	
		Fisher's LSD	**con vs TG (t20)**			**0.030***	
		Fisher's LSD	**con vs TG (t55)**			**0.418***	
	total vertical time	unpaired t-test	con vs TG	t=-1.132	1, 22	0.27	3D
	% time in center	unpaired t-test	con vs TG	t=-0.608	1, 22	0.55	S5A
elevated plus maze	% time in open arm	unpaired t-test	con vs TG	t=0.819	1, 17	0.424	S5B
	% time in closed arm	unpaired t-test	con vs TG	t=-0.915	1, 17	0.373	
forced swim	time immobile	repeated measures ANOVA	F1- genotype	F=0.816	1, 22	0.376	3E
			F2- time	**F=52.578**	**5, 110**	**<0.001*** **	
			(F1xF2)	F=1.084	5, 110	0.373	
	total time immobile	unpaired t-test	con vs TG	t=0.904	1, 22	0.376	3F
tail suspension	time immobile	repeated measures ANOVA	F1- genotype	F=0.363	1, 17	0.555	S5C
			F2- time	**F=16.215**	**5, 85**	**<0.001*** **	
			(F1xF2)	F=1.658	5, 85	0.153	
	total time immobile	unpaired t-test	con vs TG	t=0.603	1, 17	0.555	S5D
social interaction	total time at box	2-way ANOVA	F1-genotype	F=1.0	1, 44	0.323	3G/H
			F2-socialness	**F=74.51**	**1, 44**	**<0.001*** **	
			(F1xF2)	**F=4.647**	**1, 44**	**0.037***	
		Fisher's LSD	**con vs TG (strang.)**			**0.031***	
		Fisher's LSD	con vs TG (empty)			0.274	
water t maze	% correct during acquisition	repeated measures ANOVA	F1- genotype	F=0.266	1, 22	0.611	3I
			F2- time	**F=21.528**	**3, 66**	**<0.001*** **	
			(F1xF2)	F=0.291	3, 66	0.832	
	% correct during reversal	repeated measures ANOVA	F1- genotype	F=0.0004	1, 22	0.985	
			F2- time	**F=114.080**	**2, 44**	**<0.001*** **	
			(F1xF2)	F=0.923	2, 44	0.405	
operant learning & extinction	chained responses during training	repeated measures ANOVA	F1- genotype	F=0.015	1, 22	0.904	S5E
			F2- time	**F=56.086**	**4, 88**	**<0.001*** **	
			(F1xF2)	F=1.644	4, 88	0.17	
	chained responses during extinction	repeated measures ANOVA	F1- genotype	F=0.119	1, 22	0.733	
			F2- time	**F=150.388**	**2, 44**	**<0.001*** **	
			(F1xF2)	F=0.060	2, 44	0.942	
	unchained responses during training	repeated measures ANOVA	F1- genotype	F=0.155	1, 22	0.698	3J
			F2- time	F=1.532	4, 88	0.2	
			(F1xF2)	F=0.763	4, 88	0.552	
	unchained responses during extinction	repeated measures ANOVA	F1- genotype	F=0.125	1, 22	0.727	
			F2- time	**F=27.167**	**2, 44**	**<0.001*** **	
			(F1xF2)	F=0.099	2, 44	0.906	
startle sensitivity	startle response	repeated measures ANOVA	F1- genotype	F=0.158	1, 22	0.695	S5F
			F2- db	**F=28.582**	**10, 220**	**<0.001*** **	
			(F1xF2)	F=0.427	10, 220	0.932	
contextual fear conditioning	% freezing total: baseline	unpaired t-test	con vs TG	t=0.628	1, 22	0.537	S5G
	% freezing total: test	unpaired t-test	con vs TG	t=0.865	1, 22	0.396	
	% freezing per minute in test phase	repeated measures ANOVA	F1- genotype	F=0.748	1, 22	0.396	S5H
			F2- time	**F=4.445**	**2, 44**	**0.017***	
			(F1xF2)	**F=3.401**	**2, 44**	**0.042***	
		Fisher's LSD	con vs TG (min1)			0.072	
		Fisher's LSD	con vs TG (min2)			0.39	
		Fisher's LSD	con vs TG (min3)			0.648	
metabolic chambers	horizontal activity	repeated measures ANOVA	F1- genotype	F=3.531	1, 14	0.081	S5I
			F2- time	**F=45.495**	**11, 154**	**<0.001*** **	
			(F1xF2)	F=1.655	11, 154	0.089	
	vertical activity	repeated measures ANOVA	F1- genotype	F=1.347	1, 14	0.265	S5J
			F2- time	**F=41.04**	**11, 154**	**<0.001*** **	
			(F1xF2)	F=1.187	11, 154	0.3	

Table S4: Statistics for behavioral phenotyping of *Drd1a/Pet1*-silenced mice, Related to Figures 3 and S5
Statistical values are provided for *Drd1a/Pet1*-silenced behavioral phenotyping data. The figure numbers are provided to reference corresponding graphs in Figures 3 (unshaded values) and S3 (shaded values).

Table S5: Statistics for behavioral phenotyping of *Drd2/Pet1*-silenced mice

Behavioral Test	Measurement	Statistical Test	Comparison	F	df	p	Fig.
open field	ambulatory distance	repeated measures ANOVA	F1- genotype	F=16.147	1, 33	<0.001***	3A'
			F2- time	F=80.462	11, 363	<0.001***	
			(F1xF2)	F=1.774	11, 363	0.0569	
	total amb. distance	unpaired t-test	con vs TG	t=-4.018	1, 33	<0.001***	3B'
	vertical time	repeated measures ANOVA	F1- genotypes	F=8.863	1, 33	0.005**	3C'
			F2- time	F=1.853	11, 363	0.048*	
			(F1xF2)	F=2.290	11, 363	0.009**	
		Fisher's LSD	con vs TG (t30)			0.021*	
		Fisher's LSD	con vs TG (t50)			<0.001***	
		Fisher's LSD	con vs TG (t55)			<0.001***	
		Fisher's LSD	con vs TG (t60)			<0.001***	
	total vertical time	unpaired t-test	con vs TG	t=-2.982	1, 33	0.005**	3D'
	% time in center	unpaired t-test	con vs TG	t=-1.864	1, 33	0.071	S5A'
elevated plus maze	% time in open arm	Mann Whitney test	con vs TG			0.522	S5B'
	% time in closed arm	Mann Whitney test	con vs TG			0.5	
forced swim	time immobile	repeated measures ANOVA	F1- genotype	F=9.723	1, 33	0.004**	3E'
			F2- time	F=57.669	5, 165	<0.001***	
			intera. (F1xF2)	F=1.088	5, 165	0.369	
	total time immobile	unpaired t-test	con vs TG	t=3.118	1, 33	0.004**	3F'
tail suspension	time immobile	repeated measures ANOVA	F1- genotype	F=1.306	1, 33	0.261	S5C'
			F2- time	F=64.073	5, 165	<0.001***	
			(F1xF2)	F=0.362	5, 165	0.874	
	total time immobile	unpaired t-test	con vs TG	t=1.143	1, 33	0.261	S5D'
social interaction	total time at box	2-way ANOVA	F1-genotype	F=0.883	1, 66	0.351	3G'/H'
			F2-socialness	F=36.91	1, 66	<0.001***	
			(F1xF2)	F=0.458	1, 66	0.501	
water t maze	% correct during acquisition	repeated measures ANOVA	F1- genotype	F=12.389	1, 31	0.001**	3I'
			F2- time	F=81.055	7, 217	<0.001***	
			(F1xF2)	F=1.989	7, 217	0.058	
	% correct during reversal	repeated measures ANOVA	F1- genotype	F=5.884	1, 31	0.021*	
			F2- time	F=122.906	3, 93	<0.001***	
			(F1xF2)	F=3.559	3, 93	0.017**	
		Fisher's LSD	con vs TG (R2)			0.002**	
		Fisher's LSD	con vs TG (R3)			0.010*	
operant learning & extinction	chained responses during training	repeated measures ANOVA	F1- genotype	F=1.478	1, 33	0.233	S5E'
			F2- time	F=56.541	4, 132	<0.001***	
			(F1xF2)	F=1.546	4, 132	0.193	
	chained responses during extinction	repeated measures ANOVA	F1- genotype	F=0.197	1, 33	0.66	
			F2- time	F=98.012	2, 66	<0.001***	
			(F1xF2)	F=0.004	2, 66	0.996	
	unchained responses during training	repeated measures ANOVA	F1- genotype	F=6.171	1, 33	0.018*	5J'
			F2- time	F=4.131	4, 132	0.004*	
			(F1xF2)	F=2.497	4, 132	0.046*	
		Fisher's LSD	con vs TG (T4)			<0.001***	
	unchained responses during extinction	repeated measures ANOVA	F1- genotype	F=0.802	1, 33	0.377	
			F2- time	F=33.582	2, 66	<0.001***	
			(F1xF2)	F=0.221	2, 66	0.803	

Table S5 (Cont.): Statistics for behavioral phenotyping of *Drd2/Pet1*-silenced mice

startle sensitivity	startle response	repeated measures ANOVA	F1- genotype	F=0.006	1, 33	0.938	S5F'
			F2- db	F=44.395	10, 330	<0.001***	
			(F1xF2)	F=0.341	10, 330	0.969	
contextual fear conditioning	% freezing total: baseline	unpaired t-test	con vs TG	t=0.056	1, 32	0.956	S5G'
	% freezing total: test	unpaired t-test	con vs TG	t=-0.242	1, 32	0.811	
	% freezing per minute in test phase	repeated measures ANOVA	F1- genotype	F=0.058	1, 32	0.811	S5H'
			F2- time	F=1.206	2, 64	0.306	
			intera. (F1xF2)	F=4.144	2, 64	0.02*	
		Fisher's LSD	con vs TG (min1)			0.111	
		Fisher's LSD	con vs TG (min2)			0.52	
		Fisher's LSD	con vs TG (min2)			0.743	
metabolic chambers	horizontal activity (XAMB)	repeated measures ANOVA	F1- genotype	F=1.227	1, 30	0.277	S5I'
			F2- time	F=49.145	11, 330	<0.001***	
			intera. (F1xF2)	F=1.389	11, 330	0.176	
	vertical activity (ZTOT)	repeated measures ANOVA	F1- genotype	F=0.223	1, 30	0.64	S5J'
			F2- time	F=43.287	11, 330	<0.001***	
			intera. (F1xF2)	F=0.352	11, 330	0.973	

Table S5: Statistics for behavioral phenotyping of *Drd2/Pet1*-silenced mice, Related to Figures 3 and S5
Statistical values are provided for *Drd2/Pet1*-silenced behavioral phenotyping data. The figure numbers are provided to reference corresponding graphs in Figures 3 (unshaded values) and S5 (shaded values). The apostrophe denotes *Drd2/Pet1*-silenced data.

Table S6. Select transcript expression in GFP+ neurons of adult *Drd1a::cre, Pet1::Flpe, RC::FrePe* mice.

	Mouse 1							Mouse 2								Mouse 3							
Cell / Gene	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Drd1a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Drd1a	-	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Drd2	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Drd2	+	-	+	-	-	-	-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Tph2	NQ	NQ	NQ	NQ	NQ	NQ	NQ	+	+	+	+	+	-	+	-	-	-	+	+	+	+	-	+
Tph2	+	+	+	+	+	+	+	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Sert	NQ	NQ	NQ	NQ	NQ	NQ	NQ	+	+	+	+	+	-	+	-	-	-	+	+	+	+	-	+
Sert	+	+	+	+	+	+	+	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Clear = qRT-PCR, Shaded = RNAseq
"+" = CPM>1 (RNAseq) or detected (qRT-PCR)
"-" = no reads (RNAseq) or not detected (qRT-PCR)
NS = Not Sequenced
NQ = No qRT-PCR data

Table S6. Select transcript expression in GFP+ neurons of adult *Drd1a::cre, Pet1::Flpe, RC::FrePe* mice, Related to Figure 5
qRT-PCR or RNA-seq was used to assess the presence of select transcripts in *Drd1a/Pet1* GFP+ neurons (n=23). Cells were manually sorted and tested individually.

Table S7. Select transcript expression in GFP+ neurons of early postnatal (P) *Drd1a::cre, Pet1::Flpe, RC::FrePe* mice.

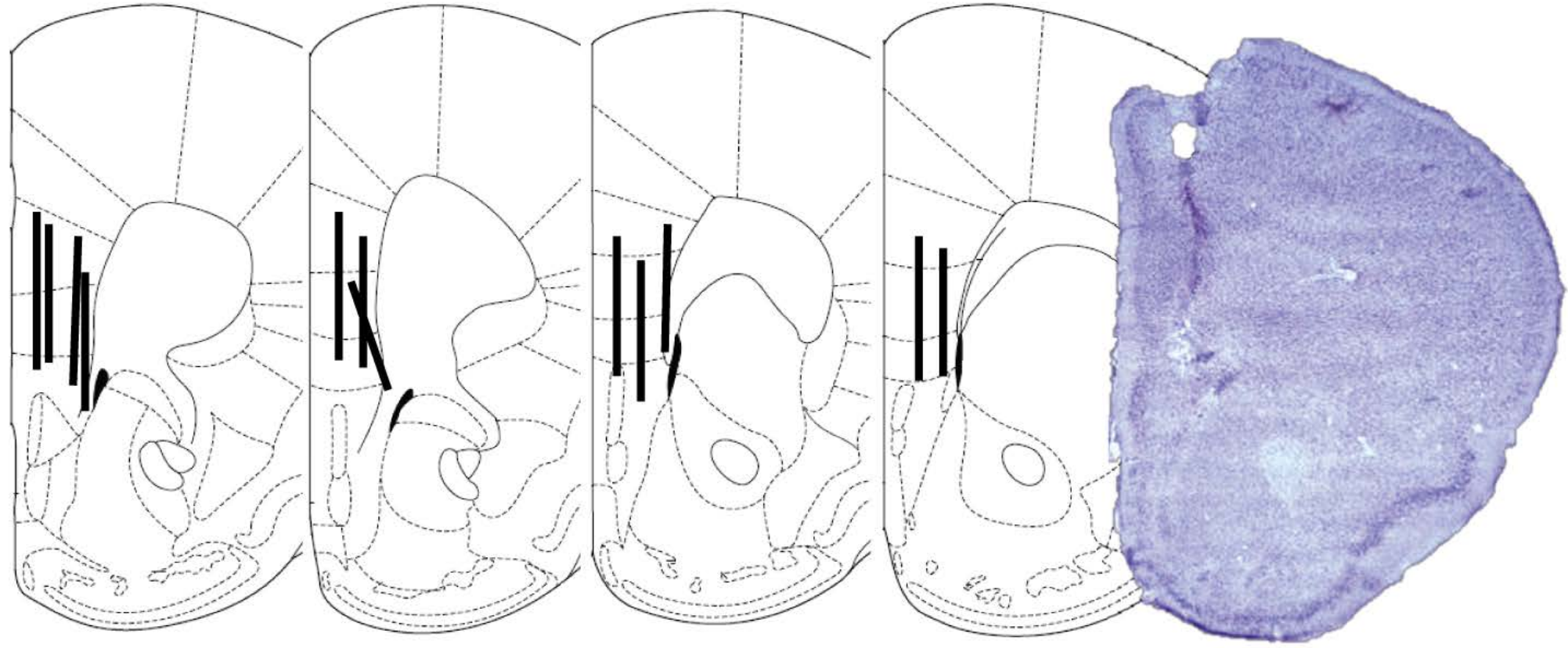
	P4				P10							
Cell / Gene	1	2	3	4	1	2	3	4	5	6	7	8
Drd1a	-	-	-	*	+	*	-	+	*	-	-	-
Drd2	-	+	+	-	-	+	-	+	*	-	*	-
Tph2	+	+	+	+	+	+	+	+	+	+	+	+
Sert	+	+	+	+	+	+	+	+	+	+	+	+
Pet1	+	+	+	+	+	+	+	+	+	+	+	+

Data is derived from RNAseq
"+" = (CPM>1)
"-" = no reads
"*" = low expression (0>CPM<1)

Table S7. Select transcript expression in GFP+ neurons of early postnatal (P) *Drd1a::cre, Pet1::Flpe, RC::FrePe* mice, Related to Figure 5
RNA-seq was used to assess the presence of select transcripts in mice at postnatal (P) mice at 4 and 10 days after birth.

Figure S1

Location of microdialoysis probes

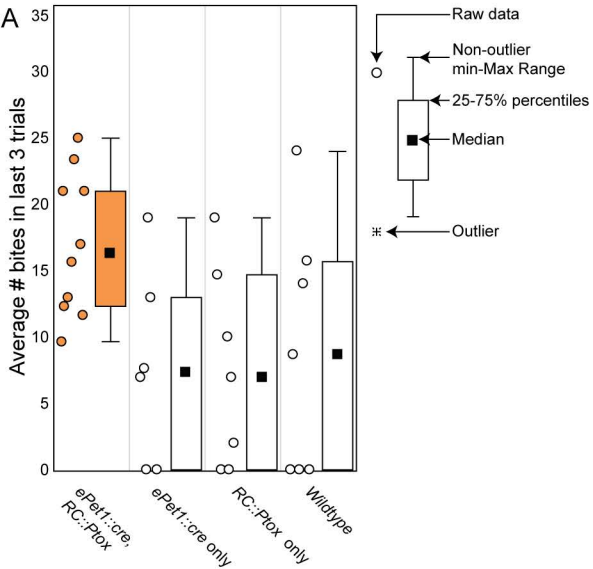


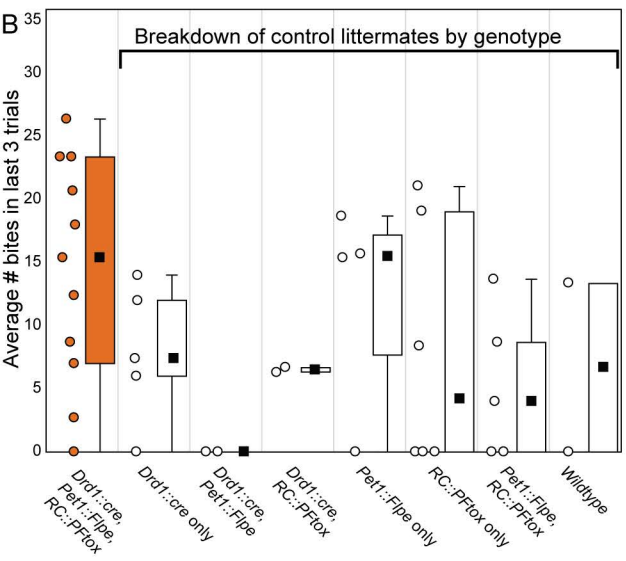
Bregma +1.94 +1.78 +1.70 +1.54

Figure S1: Microdialysis probe placement, Related to Figure 1

A) Schematics of coronal brain sections show the locations of microdialysis probe placements in the prefrontal cortex. Locations ranged from 1.54 to 1.94 mm rostral to Bregma.

Figure S2


A
Average # bites in last 3 trials
0
5
10
15
20
25
30
35
Raw data
Non-outlier
min-Max Range
25-75% percentiles
Median
Outlier
ePet1::cre,
RC::Ptox
ePet1::cre only
RC::Ptox only
Wildtype


B
Breakdown of control littermates by genotype
Average # bites in last 3 trials
0
5
10
15
20
25
30
35
Drd1::cre,
Pet1::Flpe,
RC::PFtox
Drd1::cre only
Drd1::cre,
Pet1::Flpe
Drd1::cre,
RC::PFtox
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RC::PFtox only
Pet1::Flpe,
RC::PFtox
Wildtype

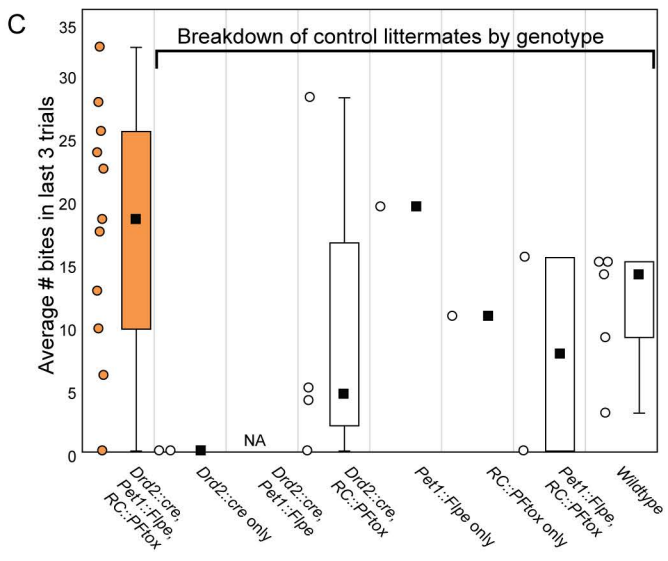
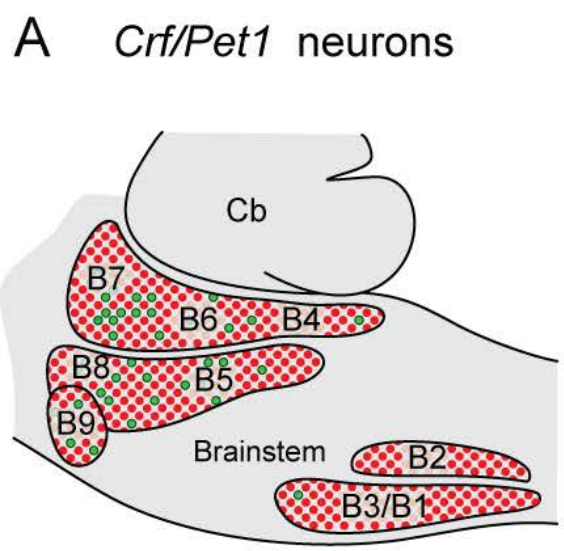

C
Breakdown of control littermates by genotype
Average # bites in last 3 trials
0
5
10
15
20
25
30
35
NA
Drd2::cre,
Pet1::Flpe,
RC::PFtox
Drd2::cre only
Drd2::cre,
Pet1::Flpe
Drd2::cre,
RC::PFtox
Pet1::Flpe only
RC::PFtox only
Pet1::Flpe,
RC::PFtox
Wildtype

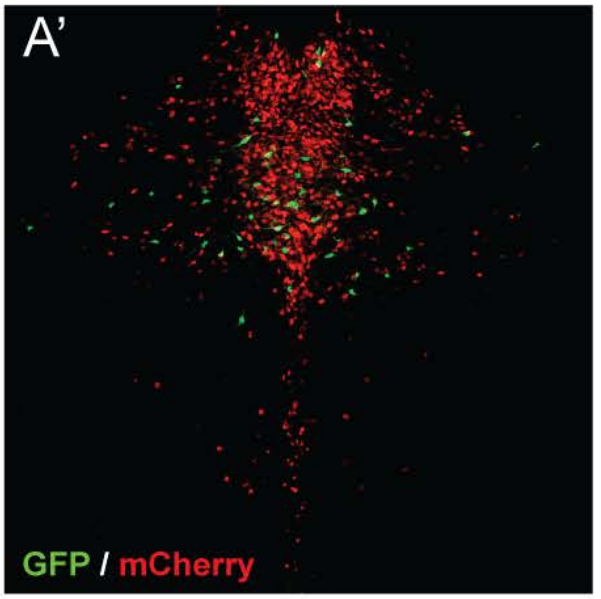
Figure S2. Plot of attack bites demonstrated during the resident-intruder assay by the sibling, non-tox-expressing controls broken down by genotype for the *Pet1*-silenced, *Drd1a/Pet1*-silenced, and *Drd2/Pet1*-silenced cohorts, Related to Figure 2

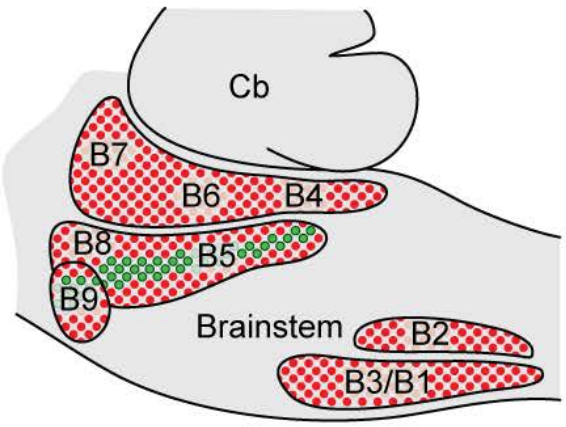
A) The graph plots the average number of attack bites demonstrated in the last three trials of the resident-intruder assay by *Pet1*-silenced animals (orange circles; tox-expressing) as well as control (non-tox-expressing) littermates of the three possible genotypes (white circles). A box plot summarizes the data raw data shown on the left of each column.

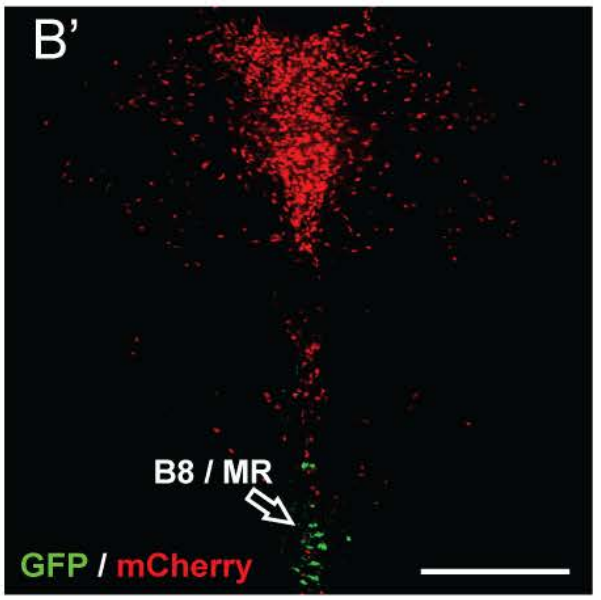
B, C) The graphs plot the average number of attack bites demonstrated in the last three trials of the resident-intruder assay by silenced animals (orange circles) as well as by control littermates (white circles) broken down by genotype for the (B) *Drd1a/Pet1* and (C) *Drd2/Pet1* cohorts. A box plot summarizes the data raw data shown on the left of each column. All silenced animals carried three transgenes including *cre, Flpe,* and *RC::PFtox,* resulting in cell-subtype-specific tox expression, while control (non-tox-expressing) animals are subdivided based on whether they carried one, two, or no transgenes.

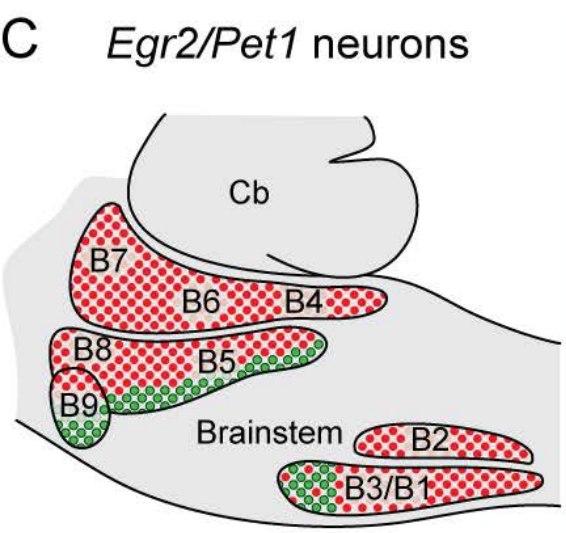
Figure S3

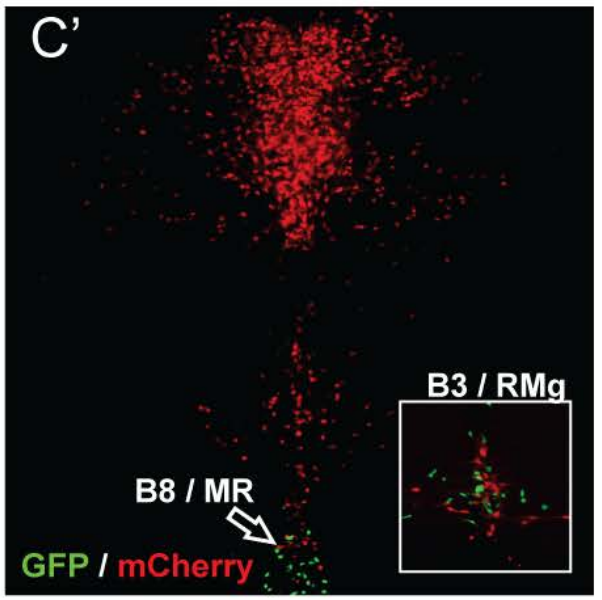

A
Crf/Pet1 neurons
Cb
B7
B6
B4
B8
B5
B9
Brainstem
B2
B3/B1

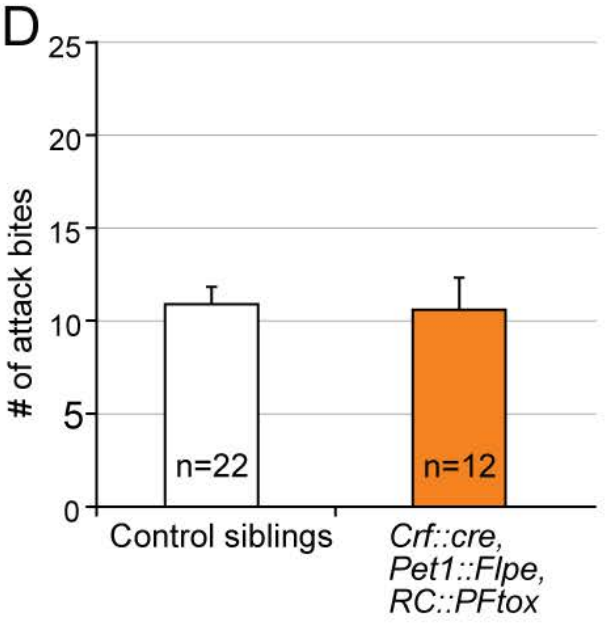

A'
GFP / mCherry

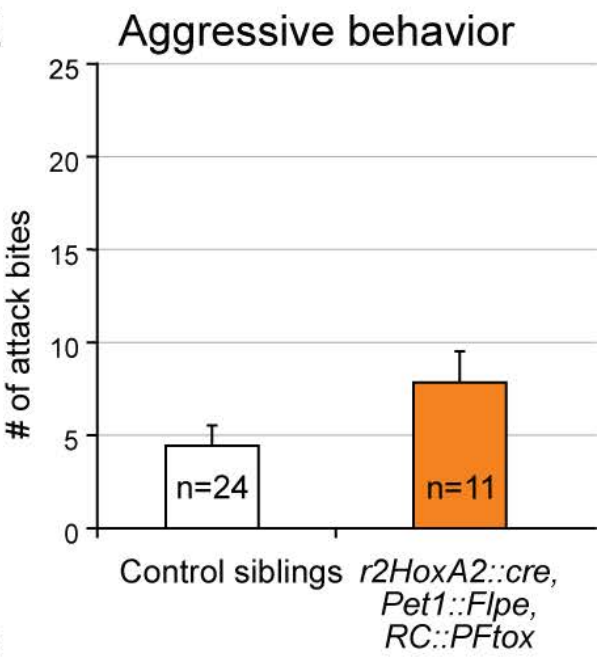

B
r2HoxA2/Pet1 neurons
Cb
B7
B6
B4
B8
B5
B9
Brainstem
B2
B3/B1


B'
B8 / MR
GFP / mCherry


C
Egr2/Pet1 neurons
Cb
B7
B6
B4
B8
B5
B9
Brainstem
B2
B3/B1


C'
B3 / RMg
B8 / MR
GFP / mCherry


D
# of attack bites
25
20
15
10
5
0
n=22
n=12
Control siblings
Crf::cre,
Pet1::Flpe,
RC::PFtox


E
Aggressive behavior
# of attack bites
25
20
15
10
5
0
n=24
n=11
Control siblings
r2HoxA2::cre,
Pet1::Flpe,
RC::PFtox

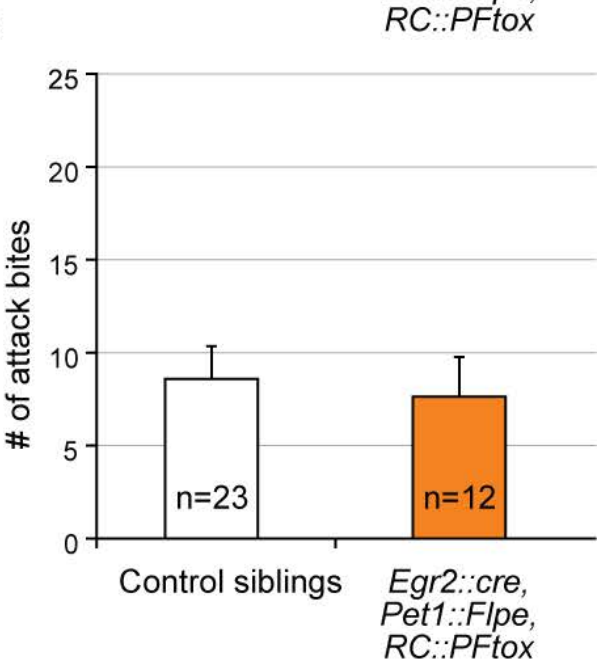

F
# of attack bites
25
20
15
10
5
0
n=23
n=12
Control siblings
Egr2::cre,
Pet1::Flpe,
RC::PFtox

Figure S3: Serotonergic neuron subtypes that fail to modulate aggression, Related to Figure 2

A/A' - C/C') For *r2HoxA2/Pet1* (A/A'), *Egr2/Pet1* (B/B'), and *Crf/Pet1* (C/C') neurons, cartoons of sagittal brain sections (A-C) illustrate the general distribution of intersectionally targeted neurons throughout the raphe system and photomicrographs show GFP+ (intersectional) and mCherry+ (subtractive 5-HT neurons) labeling (using the *RC::FrePe* reporter) in a coronal section through the dorsal raphe (inset in C' shows the raphe magnus, RMg). Cb = cerebellum. Scale bar = 50 μm.

D-F) The average number of attack bites are shown for *Crf/Pet1*-silenced (D), *HoxA2/Pet1*- (E), and *Egr2/Pet1*- (F) mice and the respective control siblings. Silencing of these three cell populations did not alter aggression.

Figure S4

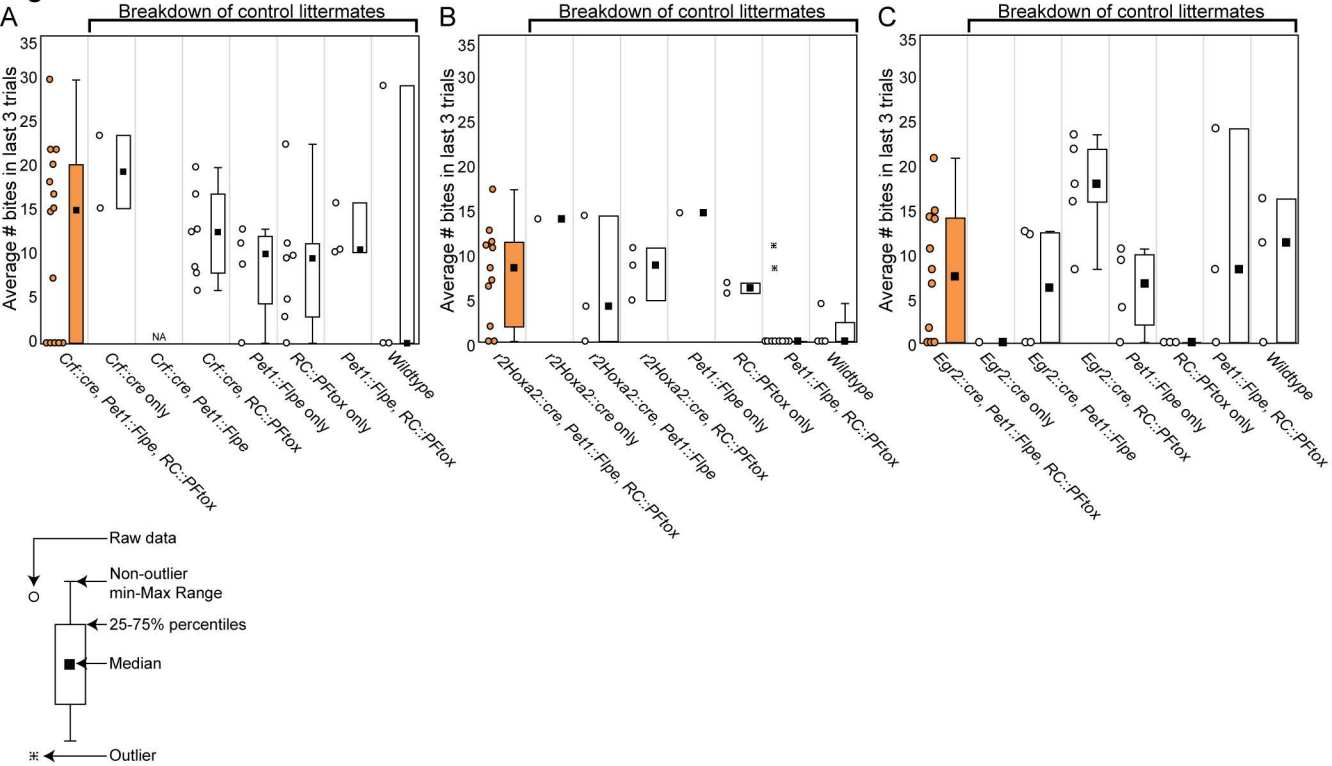

A
Breakdown of control littermates
Average # bites in last 3 trials
NA
Crf::cre, Pet1::Flpe, RC::PFtox
Crf::cre only
Crf::cre, Pet1::Flpe
Crf::cre, RC::PFtox
Pet1::Flpe only
RC::PFtox only
Pet1::Flpe, RC::PFtox
Wildtype
B
Breakdown of control littermates
Average # bites in last 3 trials
r2Hoxa2::cre, Pet1::Flpe, RC::PFtox
r2Hoxa2::cre only
r2Hoxa2::cre, Pet1::Flpe
r2Hoxa2::cre, RC::PFtox
Pet1::Flpe only
RC::PFtox only
Pet1::Flpe, RC::PFtox
Wildtype
C
Breakdown of control littermates
Average # bites in last 3 trials
Egr2::cre, Pet1::Flpe, RC::PFtox
Egr2::cre only
Egr2::cre, Pet1::Flpe
Egr2::cre, RC::PFtox
Pet1::Flpe only
RC::PFtox only
Pet1::Flpe, RC::PFtox
Wildtype
Raw data
Non-outlier
min-Max Range
25-75% percentiles
Median
Outlier

Figure S4. Breakdown of attack bites demonstrated during the resident-intruder assay by control littermate genotypes acting as comparators for the *Crf/Pet1*-silenced, *r2Hoxa2/Pet1*-silenced, and *Egr2/Pet1*-silenced cohorts, Related to Figure 2

A, B, C) The graphs plot the average number of attack bites demonstrated in the last three trials of the resident-intruder assay by silenced animals (orange circles) as well as by control littermates (white circles) broken down by genotype for the *Crf/Pet1* (A), *r2Hoxa2/Pet1* (B), and *Egr2/Pet1* (C) overall cohorts. A box plot summarizes the data raw data shown on the left of each column. All silenced animals carried three transgenes including *cre, Flpe,* and *RC::PFtox,* resulting in cell-subtype-specific tox expression, while control (non-tox-expressing) animals are subdivided based on whether they carried one, two, or no transgenes.

Figure S5

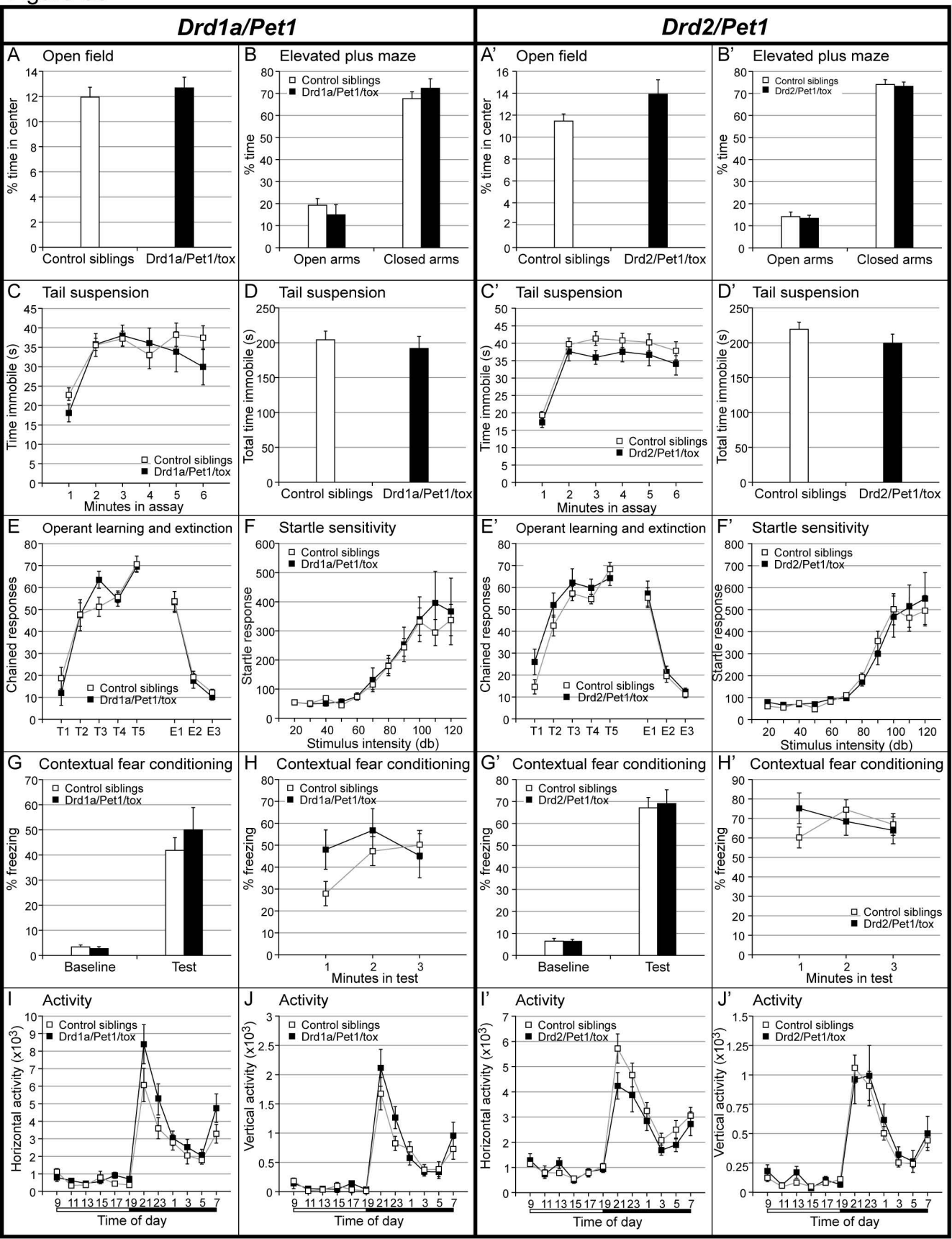

Drd1a/Pet1
Drd2/Pet1
A Open field
% time in center
Control siblings
Drd1a/Pet1/tox
B Elevated plus maze
% time
Open arms
Closed arms
A' Open field
Drd2/Pet1/tox
B' Elevated plus maze
C Tail suspension
Time immobile (s)
Minutes in assay
D Tail suspension
Total time immobile (s)
C' Tail suspension
D' Tail suspension
E Operant learning and extinction
Chained responses
T1 T2 T3 T4 T5 E1 E2 E3
F Startle sensitivity
Startle response
Stimulus intensity (db)
E' Operant learning and extinction
F' Startle sensitivity
G Contextual fear conditioning
% freezing
Baseline
Test
H Contextual fear conditioning
Minutes in test
G' Contextual fear conditioning
H' Contextual fear conditioning
I Activity
Horizontal activity (x10³)
Time of day
J Activity
Vertical activity (x10³)
I' Activity
J' Activity

Figure S5: Non-significant results from broad behavioral screening of *Drd1a/Pet1*- and *Drd2/Pet1*-silenced mice, Related to Figure 3

All values shown are mean ± SEM. For each test metric, data from *Drd1a/Pet1*-silenced mice are shown on the left (A-J), and data from *Drd2/Pet1*-silenced mice are shown on the right (A'-J'). Filled (black) boxes/bars represent *Drd1a/Pet1*-silenced or *Drd2/Pet1*-silenced animals, and open (white) boxes/bars represent respective littermate controls. Statistical measures are provided in Tables S4 (*Drd1a/Pet1*) and S5 (*Drd2/Pet1*).

A/A') **Open field.** No differences were observed in the average percent time spent in the center of the open field.
B/B') **Elevated plus maze.** No differences were observed in the percent of time spent in open or closed arms.
C/C' and D/D') **Tail suspension test.** No genotype differences were observed in the amount of time spent immobile as measured at one minute intervals (C/C') or in total (D/D').
E/E') **Operant learning task.** No genotype differences were observed in chained responses, responses linked to conditioning stimuli, either during training or during the extinction phases.
F/F') **Acoustic startle.** No genotype differences were observed in the intensity of the startle response.
G/G' and H/H') **Contextual fear conditioning.** No genotype differences were observed in the percent of time engaged in freezing behavior under baseline or conditioned trials across the entire test (G/G') or within individual one minute bins (H/H').
I/I' and J/J') **Home cage activity**. No genotype differences were observed in either horizontal (I/I') or vertical (J/J') activity in the home cage as measured over a 24 hour time period and recorded in 2-hour bins.

Figure S6

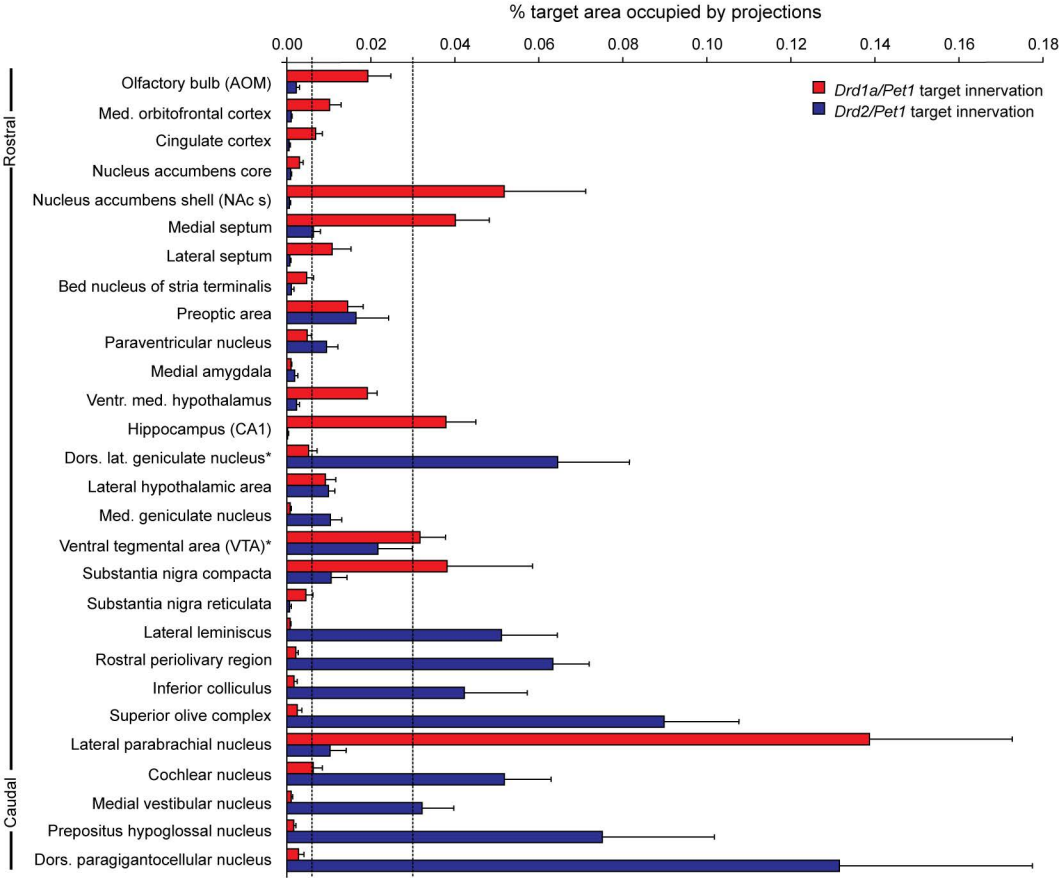

% target area occupied by projections
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
Drd1a/Pet1 target innervation
Drd2/Pet1 target innervation
Rostral
Caudal
Olfactory bulb (AOM)
Med. orbitofrontal cortex
Cingulate cortex
Nucleus accumbens core
Nucleus accumbens shell (NAc s)
Medial septum
Lateral septum
Bed nucleus of stria terminalis
Preoptic area
Paraventricular nucleus
Medial amygdala
Ventr. med. hypothalamus
Hippocampus (CA1)
Dors. lat. geniculate nucleus*
Lateral hypothalamic area
Med. geniculate nucleus
Ventral tegmental area (VTA)*
Substantia nigra compacta
Substantia nigra reticulata
Lateral leminiscus
Rostral periolivary region
Inferior colliculus
Superior olive complex
Lateral parabrachial nucleus
Cochlear nucleus
Medial vestibular nucleus
Prepositus hypoglossal nucleus
Dors. paragigantocellular nucleus

Figure S6: Quantification of GFP+ labeled axon terminals in brains of *Drd1a/Pet1* and *Drd2/Pet1* mice, Related to Figure 6
For each brain region analyzed, the average ± SEM percent of image area covered by immuno-stained puncta as labeled by *RC::FPSit* is shown for the *Drd1a/Pet1* (Red) and *Drd2/Pet1* (Blue) subtypes.

Supplemental Experimental Procedures:

Mouse lines:
All *cre*-lines used are BAC transgenics except for *Egr2::cre,* which is a knock-in allele.

Breeding Strategies:
For *en masse* silencing of serotonin neurons hemizygous *ePet::cre* mice were bred to heterozygous *RC::Ptox* mice. All intersectional strategies involved the breeding of hemizygous *Pet1::Flpe* mice to *RC::FrePe*, *RC::PFtox*, or *RC::FPSit* mice; in some cases, offspring of these crosses were bred to homozygosity for the effector allele at the *Rosa26* locus and maintained as hemizygous for *Pet1::Flpe*. To generate experimental animals, mice hemizygous for *Pet1::Flpe* and heterozygous or homozygous for the effector allele were bred to animals hemizygous for one of the *cre*-drivers. The choice of male or female for each cross depended upon animal availability.

Resident-intruder assay:
For each encounter the resident's female mate and pups were removed from the resident cage and an "instigator," CFW breeder male used to prime aggressive behavior (Fish et al., 1999), was placed inside a clear protective perforated polycarbonate cylinder (12 cm x 5 cm x 5 cm) in the center of the resident's home cage for 5 minutes. Following removal of the instigator and cylinder, a male CFW intruder mouse, who had been group-housed with 3 other males, was introduced into the home cage of the resident male. The encounter was recorded until 5 minutes following the first attack bite after which the intruder was removed. A repertoire of behaviors was analyzed during the 5 minute encounter including: 1) Aggressive behaviors (attack bites (#), sideways threats (#), pursuits (#), tail rattle (s)) and 2) non aggressive behaviors (walking (s), rearing (s), digging (s), contacts (nose of resident to nose of intruder and nose of resident to anogenital region of intruder (s), auto-grooming (s)). Analyses were performed blind to genotype and, for consistency, by the same trained individual.

Resident-intruder assays were designed to maximize offensive aggression by the resident. In the rare cases when the intruder bit the resident, the intruder was removed immediately and replaced with another group-housed male. Resident-intruder encounters were performed in the dark period (8pm -11pm) under red light conditions of a standard 12/12 light/dark cycle (light from 6am to 6pm).

5-HT microdialysis after dexfenfluramine injection:
Four double transgenic *ePet::cre, RC::Ptox* mice and 8 control littermates (4 *ePet::cre*, 4 *RC::Ptox*) were implanted with a cannula for a microdialysis probe in the mPFC for 5-HT measurement. Microdialysis guide cannulae (CMA 7, Harvard Apparatus, Holliston, MA) targeting the prelimbic and infralimbic regions of the mPFC at AP +2.0mm from bregma, ML – 0.3 mm, DV – 1.0 mm from dura. Animals were allowed 4-7 days of recovery before testing. The night before the test day, mice were anesthetized with inhaled isoflurane (Webster Veterinary, Devens, MA) to insert the microdialysis probe with a 1-mm active membrane (CMA 7, Harvard Apparatus) into the mPFC. ACSF infused at overnight flow rate of 0.5 µl/min. On the test day, the flow rate was increased to 1.5 µl/min for 1 hr before dialysate sample collection. Four baseline samples preceded an i.p. injection of sterile saline (1ml/100g), before a final i.p. injection of 3 mg/kg dexfenfluramine (Tocris) 20 min later. The dose was chosen based on previous microdialysis studies demonstrating a transient rise of extracellular 5-HT in mice and rats (Knobelman et al., 2000; Laferrere and Wurtman, 1989; Rocher and Gardier, 2001).

5-HT was measured using electrochemical detection equipped with high performance liquid chromatography (Hwa et al., 2016). A stabilizing agent of 20 mM phosphate buffer with 25 mM ethylendetaminetetraacetic acid (EDTA; 7.5 µl) was added to 30 µl dialysate samples. The mobile phase consisted of 150 mM ammonium acetate, 50 mM citric acid, 27 µM EDTA, 10% methanol, and 1% acetonitrile with pH adjusted to 4.6. 5-HT was separated by a cation-exchange column (Capcell Pak SDX, 1.5mm x 250 mm, 5 um I.D., Shiseido, Tokyo, Japan) at 30°C and a flow rate of 0.2 ml/min. Standard curves with known amounts of 5-HT in a range of 0.125-0.5 pg were used to determine 5-HT concentrations. The limit of detection was 2 fg under these conditions with a 9.8% recovery rate.

Mouse lines: Generation of *RC::FPSit* mouse:
A cassette containing Synaptophysin-GFP (Addgene, plasmid 26084: pTRE-Bi-SG-T), IRES-tdTomato-WPRE (Clontech; PT5062-5) and a rabbit beta-globin polyA sequence were cloned into the multiple cloning site of our pFPH plasmid. 5' to this multiple cloning site, pFPH contains an *FRT*- flanked cassette consisting of the *PKG-neo* sequence (for positive selection of homologous recombinants) and the lox^2 transcriptional stop cassette derived from pBS302 (Sauer and Durre, 1993), followed by a *lox*P-flanked cassette containing a concatemer of SV40pA stop sequences (Dymecki and Kim, 2007). The resulting vector was referred to as pFPH-SP-GFP-IRES-tdTomato-WPRE-pA. The DNA region containing the two stop cassettes and SP-GFP-IRES-tdTomato-WPRE was excised from pFPH-SP-GFP-IRES-tdTomato-WPRE-pA using PacI and AscI sites and subcloned into the AsiSI and AscI sites of a CAG-MCS vector. The resulting vector was designated CAG- FPH-SP-GFP-IRES-tdTomato-WPRE-pA. Once validated in cell culture for recombination efficacy, the fragment containing the CAG sequences, two stop cassettes, and SP-GFP-IRES-tdTomato-WPRE-pA was excised using PacI and AscI and subcloned into the PacI and AscI sites of pRosa26-1 (gift from Dr. Philippe Soriano). The completed targeting vector, pR26-CAG- FPH-SP-GFP-IRES-tdTomato-WPRE-pA was linearized and electroporated into ES (Tc-1) cells. The

resulting G418 resistant colonies screened by PCR for homologous recombination at the *R26* locus (Zambrowicz et al., 1997) as described previously (Ray et al., 2011) and verified by genomic Southern blot analysis. Using standard methods, ES cells from a single recombinant ES clone were used to derive *RC-FPSit* chimeric mice. Germline derivation was achieved by crossing to C57BL/6J mice.

Broad behavioral phenotyping (described in the order the tests were performed):

Metabolic cage analysis:

Mouse metabolic analyses were performed using the Comprehensive Lab Animal Monitoring System (CLAMS) from Columbus Instruments. Mice were individually placed in CLAMS chambers (plastic cages, 20 cm x 16 cm) for a period of three consecutive days. The first two days allowed for the requisite familiarization of the mice to the chambers. Behavioral measures (food intake and locomotor activity) were only analyzed during the final 24 h. XAMB and ZTOT were used as measures for horizontal and vertical activity, respectively. In these chambers, mice had free access to food (regular chow) and water. After this test, mice were returned to their home cage.

Open field:

The open field apparatus consisted of a 27.9 cm × 27.9 cm, clear Plexiglas arena equipped with three 16-beam infrared arrays (Med Associates). Mice were acclimated to the experimental test room for at least 30 minutes prior to testing. To start a session, a mouse was placed into the center of the arena and allowed to freely explore for a total of 60 minutes. The total distance traveled (centimeters), time spent in center (12.7 cm x 12.7 cm) (s) and vertical beam breaks (an indication of rearing activity) were recorded automatically.

Elevated plus maze:

The elevated plus-maze apparatus consisted of two open and two closed arms extended out from a central platform. Each arm of the maze was 30 cm long and 5 cm wide. The maze surface was 85 cm above the floor. Each mouse was placed in the same position on the open arm of the maze at the beginning of the assay, facing the center, and allowed to explore the apparatus for 5 minutes. A computer-assisted video-tracking system (TopScan software, CleverSys Inc.) was used to record the number of open and closed arm entries as well as the total time spent in open, closed, and center compartments.

Tail suspension:

An automated Tails Suspension Test device (Med Associates) was used to measure the duration of behavioral immobility. The automated device consisted of a box-like enclosure (box size: 32 x 33 x 33 cm) that was open on the front side. A vertical aluminum bar (bar size: 11.5 x 2.2 x 0.15 cm), suspended from the top, was connected to a strain gauge that detected any movements by the mouse. Mice were suspended by the tail with tape for 6 minutes. The total duration of immobility was calculated as the time the force of the mouse's movements was below a predetermined threshold. The following settings were used in all experiments: threshold =2, gain = 4, resolution 10 ms, starting trigger = 10. Eight experimental boxes were used simultaneously in this study.

Social interaction test:

A three-chambered rectangular apparatus (62 cm x 40 cm) was used to evaluate sociability and preference for social novelty as previously described (Nadler et al., 2004). Test mice were first placed in the middle chamber and allowed to explore all three chambers for ten minutes (habituation). After the habituation period, the test mouse was confined to the center chamber while an unfamiliar male mouse (C57BL/6J) (stranger), that had no prior contact with the subject mouse, was confined to a random, counterbalanced side chamber in a small perforated container. The test mouse was then allowed to explore the entire social test apparatus for a ten-minute session. The amount of time spent in each chamber and the time spent in close proximity to either perforated container, which was defined as the area within 2.5 cm around the perforated container, were scored by an automated video-tracking system (TopScan; Cleversys).

Forced swim:

Mice were placed for 6 minutes in a glass cylinder (height: 35 cm; diameter: 17 cm) filled with water (25 ± 1 °C) to a depth of 25 cm. The water depth was adjusted so that the animals were forced to swim or float without their hind limbs or tail touching the bottom. The session was videotaped and analyzed afterwards on the computer blind to genotype. Duration of immobility (the time during which the subject made only the small movements necessary to keep their heads above water) was scored by a trained observer.

Startle sensitivity:

All subjects were initially placed into a restrictive holder (i.e. acrylic cylinder with a 3.2 cm internal diameter) that allowed slight changes in movement to be closely detected and controlled. Each animal holder was placed into an individual acoustic chamber (Med Associates) on top of a transducer platform, which measured the active response of the subject to both weak and startle stimuli. Animals were submitted to sessions consisting of 10 blocks of 11 trials each. Within each block, various acoustic stimuli ranging from 20 to 120 dB were presented in a random order with a variable inter-trial interval with an average of 15 s (10-20 s). The duration of the stimulus was 40 ms. Responses were recorded for 150 ms from startle onset and are sampled every msec. Mice were placed back into the home cage immediately after testing.

Operant learning and extinction:
Five days prior to the start of operant conditioning experiments, all subjects were placed on a food restriction diet. On the first day, baseline body weights were recorded and mice were individually placed into clean cages with access to water only. Food rations (regular chow) were calculated as a function of each individual's bodyweight loss/gain from the previous day and delivered daily to maintain a stable 80–85% of free feeding weight. During the 5-day period preceding training, mice were given fifteen 20 mg casein pellets (BioServ) daily, habituating the mice to the rewards. The food restriction procedure was maintained for the entire training and testing period.

The testing apparatus was a standard sized operant chamber (Med Associates) with plexiglas side-walls, stainless steel end-walls and a steel-bar floor. One pellet receptacle connected to a silent pellet dispenser was located in the center of one end-wall with a small yellow stimulus light located directly above the receptacle. A photobeam sensor was located inside the receptacle to detect head entries. Pellet dispensers were filled as needed with 20 mg casein pellets (BioServ). A lever was placed adjacent to the receptacle and was available to the animal during acquisition and extinction sessions.

Prior to training, food-restricted mice were exposed to the operant chambers for 45 minutes without access to the operant lever. During this habituation session, the mice received 20 exposures to a 1-s light stimulus with an inter-trial interval of 120 s. Each stimulus light display was paired with the delivery of a food pellet. 24 h after the habituation session all mice began the acquisition phase, which consisted of daily 30-minute training sessions over five days. Each lever press was also paired to a light stimulus (1 s). Under continuous reinforcement (FR1 schedule), each lever press was paired with the delivery of a food pellet. Lever presses followed by a head entry into the food cup within 30 s are scored as chained responses. Learning is reflected by an increased number of chained responses across days and low level of unchained responses. Lever presses followed by another lever press were scored as unchained responses. Extinction training started at least 24 h after the last acquisition session. Daily extinction sessions were conducted over 3 days under similar conditions as during acquisition with the exception that food pellets were no longer delivered after a lever press. Extinction learning is reflected by a decrease in the number of chained responses across days. The mice were under diet restriction during the entire training period. Total lever presses, chained, and unchained responses were collected automatically by the MED-PC IV software, which allows for the control, execution, and analysis of programs written in Med State notation.

Water T maze:
The apparatus used for this paradigm was a T shaped pool (long arm: 50 x 10 x 20 cm; short arms: 25 x 10 x 20 cm each). Fresh water was added each day and animal feces were removed from the pool between trials. Water temperature was continuously monitored and maintained at 25C ±1C. To ensure that the animals could not see the submerged platform while swimming, the water was made opaque by adding non-toxic white food coloring. During water maze training, animals received 10 trials per day until both groups (controls and triple transgenics) met the criterion of 80% correct on 2 consecutive days. All trials lasted until the animal found the platform or a maximum time of 90 s was reached. After the animals reached the platform they were allowed to stay there for a maximum of 15 s before removal. Between trials animals were placed in a holding cage with a blanket to regulate body temperature. After completion of a daily session, animals were dried with paper towels before being placed back in their home cage to prevent body temperature fluctuations. Once the criterion of 80% correct on 2 consecutive days was reached, the hidden platform was switched to the opposite arm of the T and reversal training started for an additional 3 days.

Contextual fear conditioning:
The testing apparatus was a standard sized operant chamber (Med Associates) with plexiglass side walls and stainless steel end-walls. The floor consisted of steel bars 4.8 mm in diameter and spaced 1.6 cm apart in which a scrambled electric shock could be delivered. A fan was also attached to the sound attenuation chamber and was used to provide masking noise. Eight fear conditioning chambers were used in parallel and the FreezeScan® computer-assisted video tracking system was used to assess freezing behavior (CleverSys Inc.). Prior to testing, all subjects were acclimated to the testing room for at least 15 minutes. Testing occurred over three sessions separated by a 24 h delay. To begin session 1, a subject was placed in an illuminated chamber and allowed to explore for 2 minutes. At the end of this 2-minute period, a 2 s, 0.5 mA shock was delivered via the grid floor. A second, identical shock followed 2 minutes later, and the subject was subsequently given one additional minute before being removed from the chamber. During the entire session, freezing behavior (i.e. lack of displacement, body movement, head turning and grooming) was recorded via the FreezeScan® system. 24 h later, in session 2, mice received two 2 s, 0.5 mA shock shocks, the first after 3 minutes the second 2 minutes later. The subject was subsequently given one additional minute before being removed from the chamber. Session 2 was implemented due to prior experience with the chamber in the previously executed operant learning and extinction test. Session 3 occurred 24 h later and consisted of a 3-minute period during which the subject was allowed to freely explore the chamber in the absence of any foot shock. Similar to session 1, freezing behavior was recorded in session 2 and 3 using the FreezeScan® system.

Immunohistochemistry:
Tissue processing:

Anesthetized mice were perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were extracted, post-fixed in 4% paraformaldehyde at 4ºC overnight, cryoprotected in 30% sucrose/PBS, and embedded in TFM compound prior to sectioning. Tissue was sectioned on a cryostat at 40 μm and processed as free-floating sections.

Immunohistochemistry Procedures:

Detection of tox-GFP fusion protein (Figure 1B and C) and quantification of subpopulation numbers (Figure 2G). 40 μm free-floating sections were rinsed with PBS and incubated with 0.3% H2O2 in PBS for 30 minutes at RT to quench endogenous peroxidase activity. Sections were rinsed with PBS and blocked with 5% normal goat serum (NGS)/PBS/0.1% Triton-X-100 for 1 h at RT, followed by incubation with a rabbit anti-GFP (gift from Devreotes Lab) in 1% NGS/PBS/0.1%Triton for 48 h at 4ºC. Sections were rinsed with PBS three times, and incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Labs, BA-1000) in 1% NGS/PBS/0.1%Triton for 30 minutes at RT. Following three rinses with PBS, immunoreactivity was detected using the Vectastain Elite ABC kit (Vector Labs, PK-6100) and DAB (3, 3'-diaminobenzidine)(Sigma, D5637) according to the manufacturer's instructions. Sections were mounted on Superfrost Plus slides (VWR, 48311-703), dehydrated and cleared through a series of ethanol dilutions and xylene, and subsequently, and coverslipped with DPX mounting medium (Electron Microscopy Sciences, 23512). GFP+ cells were counted in every 6th section. The resulting number was multiplied by 6 to obtain the 'number (#) of GFP+ cells/brain'.

Detection of (1) serotonin/Vamp2 (Figure 1D and E), (2) GFP/mCherry (Figures 2, S3, and 4), or (3) synatophysin-GFP fusion (Figure 6). Sections were washed 3x10 min. in PBS, and then blocked for 1h in 5% donkey serum/PBS/0.1%TritonX-100, then incubated for 48h at 4ºC in primary antibody solution containing 5% donkey serum/PBS/0.1%Tritonx-100. Primary antibodies used were (1) chick anti-GFP (1:2000, Abcam, ab13970-100) and rabbit anti-Vamp2 (1:500, Synaptic Systems, #104202), (2) chick anti-GFP and rabbit anti-dsRed (1:1000, Clontech, 632496), or (3) just chick anti-GFP. After three 10 minute washes in PBS, sections were incubated in secondary antibody solution containing 2% donkey serum/PBS/0.1%Triton x-100 for 2h at room temperature (RT). Secondary antibodies used were donkey anti-chick Alexa 488 and donkey anti-rabbit Cy3 (Jackson Immuno Research, 703-545-155 and 711-165-152, respectively) as appropriate. After a final wash sections were mounted onto Superfrost Plus slides and cover-slipped with Aqua Polymount (Polysciences).

Projection mapping:

Brain tissue of three *Drd1a::cre, Pet1::Flpe, RC::FPSit* and three *Drd2::cre, Pet1::Flpe, RC::FPSit* male mice from independent litters was collected at P(postnatal day)90. 40 μm free-floating sections were stained for GFP to detect Synaptophysin-GFP and DAPI to label cell nuclei. For each sample analyzed, two adjacent 40x confocal stacks were acquired bilaterally on a Zeiss LSM780 confocal microscope (resulting in four stacks in total per target region). Target region identification was based on anatomical landmarks using the DAPI staining and was consistent across specimens. Quantification of projections within the hippocampus and olfactory bulb focused on the CA1 and AOM, respectively, as these subregions were found to be representative of innervation throughout the structures at large. Staining and imaging protocols were identical amongst the six samples analyzed.

Quantification of target innervation:

Overview. Four confocal image stacks were captured per neural region analyzed in each sample, yielding a total of 112 images per sample. Image stacks (.czi files) were imported into FIJI (http://fiji.sc/Fiji) for analysis of axon projection area. Each stack contained between 18 and 27 optical slices. Axon projection area for each serotonergic subtype was assessed via analysis by a FIJI macro in conjunction with a Matlab program, such that all images, obtained from 28 different neural regions and 6 different experimental samples, were treated identically, except where indicated. This analytic procedure is described briefly.

Background subtraction. Upon import into FIJI, each Tiff file image stack was split into the constituent GFP and DAPI channels. The first stage of analysis involved background subtraction of the GFP stack via two methods. First, the Rolling Ball technique was applied with a radius of 5 to obtain an even distribution of background signal across the image. Nuclear background was then eliminated by subtracting out regions of the GFP stack that colocalized with DAPI signal. To do so, the DAPI channel was thresholded as a stack using the Li algorithm and converted into a binary image. A subsequent binary subtraction of the DAPI binary stack from the GFP fluorescent stack yielded a background subtracted GFP stack. A Gaussian Blur Filter (sigma=1) was then applied to the background subtracted GFP stack.

Thresholding and particle counting. Background subtracted fluorescent GFP image stacks from each neural region in all six samples were thresholded with a grey scale value range extending from 50 to 255, except for images of the VTA and DLG. Images taken in the latter two regions were thresholded with a grey scale range extending from 30 to 255 due to the demonstrably weaker signal apparent in projections found within these regions. Subsequently, particle analysis was conducted: Signal above threshold constituted by two or more contiguous pixels (2-infinity) was counted as a particle. Projection signal was thus defined as any cluster of two or more pixels (a particle) with a fluorescence intensity greater than or equal to 50 (30 in the case of the VTA and DLG). The area of each defined particle was measured and exported in tabular format as a .csv file.

Data analysis. Data analysis of particle area within each image stack was performed in Matlab and consisted of four stages: (1) The 15 consecutive optical slices containing the most projection signal area were identified in each image stack. (2) The area of all particles, which met the above criteria, within those 15 consecutive optical slices was summed to obtain the cumulative area occupied

by projection signal. (3) The percent area occupied by projection signal was calculated by dividing the cumulative area occupied by projection signal by the cumulative area of 15 optical slices. (4) The percent area occupied by projection signal was averaged within images of the same neural region across samples, yielding a metric termed the "mean area occupied by projections" for the *Drd1a* and *Drd2* serotonergic subtypes.

Cell sorting, QPCR, and RNA-Seq:
Triple transgenic *Drd1a::cre, Pet1::Flpe, RC::FrePe* and *Drd2::cre, Pet1::Flpe, RC::FrePe* mice, aged P4 (2 *Drd1a::cre, Pet1::Flpe, RC::FrePe* animals), P10 (1 *Drd1a::cre, Pet1::Flpe, RC::FrePe* animal) and P49-P62 (6 animals, 3 per genotype) , were anesthetized with isoflurane and decapitated in accordance with Institutional Animal Care and Use Committee Protocols of Harvard Medical School. Fluorescently labeled cells were manually sorted as outlined in (Hempel et al., 2007). The main steps are summarized here. Brains were extracted in ice cold, oxygenated artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1 mM NaH2PO4, 25 mM NaHCO3, and 2mM CaCl2, adjusted to an osmolarity of ~320 mOsm with dextrose. ACSF also contained channel and receptor blockers which were added to prevent excitotoxicity and promote the general health of the extracted tissue, specifically: 0.1 μM tetrodotoxin (TTX) to block voltage-gated sodium channels, 50 μM D,L-2-amino-5-phosphonovaleric acid (APV) to block NMDA-receptor mediated currents, and 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) to block AMPA-receptor mediated currents. Acute 400 μM coronal brain slices encompassing the dorsal raphe (DR) were prepared on a vibratome, and digested for approximately 1 hour (25 minutes for the P10 animal) in ACSF containing 1mg/ml of protease from *streptomyces griseus* (Sigma-Aldrich). DR regions containing GFP-expressing neurons were identified under a dissection microscope with fluorescence optics, microdissected with fine scissors, and gently triturated in a series of Pasteur pipettes with decreasing diameter tip diameters to break apart digested tissue. Dissociated cells were then plated on sylgard-lined 35 mm petri dishes and GFP-labeled neurons were aspirated with a glass pipette. Sorted neurons were moved through a series of 2-3 clean dishes containing filtered ACSF to clear off cellular debris and ensure the purity of labeled cells. Finally, single cells were moved in and out of the glass pipette tip 2-4 times to further shake off any remaining debris, and then deposited in a cell lysis buffer (PicoPure RNA Isolation Kitl; Applied Biosystems), heated for 30 minutes at 42º C, and then stored at -80º C until continuing with the RNA isolation protocol. cDNA was synthesized and amplified from the harvested RNA using the Ovation RNA-Seq System v2 (Nugen). This amplified cDNA served as the template for QPCR reactions using primers for Actg (forward primer: ACCAACAGCAGACTTCCAGGAT, reverse primer: AGACTGGCAAGAAGGAGTGGTAA), which served as a housekeeping control, Tph2 (forward primer : GAGCTTGATGCCGACCAT, reverse primer: TGGCCACATCCACAAAATAC) and Sert (forward primer: CAAGTTCAACAACAACTGTTACCAA, reverse primer: TAGCCAAGCACCGTGAAGAT) which served as serotonergic marker genes, and finally Drd1a (forward primer: CCAAGAACGTGAGGGCTAAG, reverse primer: TGAGGATGCGAAAGGAGAAG), and Drd2 (forward primer: ACCACTCAAGGGCAACTG, reverse primer: TGACAGCATCTCCATTTCCAG or forward primer: TCATGAAGATCCTGCACTGC, reverse primer: GAGTCCATCTGGGCCTTTC). Reactions were prepared using the SYBR select master mix (Applied Biosystems) and run on a 7500 Fast Real-Time PCR System (Applied Biosystems). Amplified cDNA from two *Drd1a-cre, Pet1::Flpe, RC::FrePe* animals (ages P10 and P62) and one *Drd2-cre, Pet1::Flpe, RC::FrePe* animal (age P62) was also used to generate next generation sequencing libraries, prepared using the Ovation Ultralow DR Multiplex System (Nugen). Quantification and quality control of these sequencing libraries was assessed using TapeStation and Q-PCR. Libraries were then sequenced using the Illumina HiSeq 2000 platform.

Electrophysiology:
Slice preparation:
Brain slices were obtained from *Drd2::Cre, Pet1::Flpe; RC::FrePe* mice. Mice were anesthetized with isoflurane prior to sacrifice. Brains were removed and immediately placed in ice cold sucrose buffer (in mM, sucrose, 250; KCl, 3; $MgSO_4 \cdot 7H_2O$, 2; NaH_2PO_4, 12.5; dextrose, 10; $NaHCO_3$, 26; $CaCl_2$, 0.1) oxygenated with 95% O_2/5% CO_2 for a 5-10 minute incubation and then during sectioning. Sections through the rostral to caudal extent of the dorsal raphe obtained using a vibratome were transferred to a holding chamber containing artificial cerebrospinal fluid (aCSF; in mM, NaCl, 1024; KCl, 3; $MgSO_4 \cdot 7H_2O$, 2; NaH_2PO_4, 12.5; dextrose, 10; $NaHCO_3$, 26; $CaCl_2$, 2.5), incubated at 37°C for 1-hour, and then placed at room temperature until recording.

Whole cell patch clamp recordings:
Similar to prior published work (Grant et al., 2011), slices were secured in a chamber with a constant flow of aCSF (~2ml per minute) warmed to approximately 32-34°C with an inline heater. Fluorescently labeled cells were targeted for recordings. In mice with the intersectional *RC::FrePe* allele, neurons expressing both Cre (Drd2) and Flpe (Pet1) recombinase also express GFP and neurons expressing Flpe, but not Cre, express mCherry. Fluorescent proteins are not expressed in Cre only neurons or neurons with no recombinase expression. Following identification of a neuron via GFP or mCherry mediated fluorescence, neurons were visualized using differential interference contrast and patched with a glass electrode (2-6 MW starting resistance) filled with electrolyte solution (mM unless stated otherwise, K-gluconate, 130; NaCl, 5; Na Phosphocreatine, 10; $MgCl_2 \cdot 6H_2O$, 1; HEPES, 10; EGTA, 0.02; Na_2-GTP, 0.5; Mg-ATP, 2; biocytin, 1 mg/ml). Voltage clamp data were obtained using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), digitized (Digidata 1322, Molecular Devices), and recorded using Clampex 9.2 (Molecular Devices). In all experiments, the current responses to various ligands were recorded in voltage clamp with a holding potential of -60 mV. In some experiments, a ramp protocol was employed to visualize current responses over a range of potentials. Starting at -60 mV, the holding potential was dropped to -100 mV, raised linearly over 60s to -30 mV, and finally dropped back to the -60 mV holding potential.

Drugs:
Quinpirole (100 or 10 μM), dopamine (30 μM), sulpiride (1 μM), bicuculline (20 μM), DNQX (20 μM), tetrodotoxin (1 μM, Abcam). Unless stated otherwise, drugs and reagents were obtained from Sigma-Aldrich.

Statistics:
Response of GFP neurons to 100 μM quinpirole was tested with a single sample t-test against the null hypothesis ($H_0 = 0$).

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