PNAS
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Supplementary Information for

Early Adolescent Rai1 Reactivation Reverses Transcriptional and Social Interaction Deficits In a Mouse Model of Smith-Magenis Syndrome

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Datasets 1 to 3

SI Materials and Methods

Generation of the Rai1^{STOP} mice

The targeting construct for producing the *Rai1^{STOP}* allele was generated by first modifying *PGKneotpAlox2* (Addgene #13444) vector that contains *loxP* flanked neo cassette and 3X SV40 polyadenylation sequences. The 5' homology arm (3.2 kb upstream of the ATG of the *Rai1* major exon) was inserted 5' of the first *loxP* site of *PGKneotpAlox2* plasmid. A DTA cassette for negative selection was inserted 3' of the second *loxP* site. The 3' homology arm (2 kb downstream of ATG, including the *Rai1* major exon) was then cloned into a *pCR2.1-TOPOGFP* vector, a fragment was subsequently cloned into the 5' arm-containing *PGKneotpAlox2* vector. The final construct was linearized with SalI restriction enzyme and electroporated into 129Sv/SvJ ES cells. The correctly targeted clones were identified by long-range PCR and DNA sequencing. Targeted ES cells were microinjected into BL/6 blastocysts, and chimeras with successful germ line transmission were identified by PCR and were used to expand the colony. ES cell manipulations and blastocyst injections were performed by the Stanford Transgenic Research Facility.

Mouse genotyping

Genotyping for *Rai1$^{STOP/+}$* mice was performed using three primers: STOP1 5'-CATCAAGGTGAACTTCAAGATCCG-3', STOP2 5'-AGAGTCCAGATGGCACTACAGG, and STOP3 5'-TTGCCATGGAAACCACACCTT-3', for a 608-bp wild-type band and 276-bp Rai1-STOP band. Genotyping for *Ubc^{CRreERT2}*, *Vgat^{Cre}*, and *Vglut2^{Cre}* mice were performed using primers 5'- CACCCTGTTACGTATAGCCG-3' and 5'-GAGTCATCCTTAGCGCCGTA-3' for a 300-bp Cre band, and primers 5'-CCAATCTGCTCACACAGGATAGAGAGGGCAGG-3'

and 5'- CCTTGAGGCTGTCCAAGTGATTCAGGCCATCG-3' for a 500-bp internal control band. Genotyping for *Thy1*^{EGFP} mice were performed using primers 5'-CGGTGGTGCAGATGAACTT-3' and 5'- ACAGACACACACCCAGGACA-3' for a 415-bp Thy1-EGFP band, and primers 5'- CTAGGCCACAGAATTGAAAGATCT-3' and 5'-GTAGGTGGAAATTCTAGCATCATCC-3' for a 324-bp internal control band. Genotyping of *Rai1*$^{flox/flox}$ mice was performed as previously described (1).

RNA-seq, qRT-PCR, and data analysis

Cortices from adult male WT (n=5), *Rai1*$^{STOP/+}$ (n=5), and *Ubc*^{CreERT2};*Rai1*$^{STOP/+}$ (Rescue, n=5) mice (all treated with tamoxifen at 3 weeks of age) were harvested for RNA-seq experiment. Total RNA was extracted by TRizol reagent (Life Technologies) and phenol-chloroform-isoamyl alcohol (Life Technologies). The residual DNA was removed with on-column DNase digestion (Qiagen) for 30 minutes and RNA was further purified using RNeasy Kit (Qiagen). Total RNA from 5 biological replicas of each genotype was used. Ribosomal RNA was depleted with Ribo-Zero rRNA removal kits (Illumina). For quantitative RT-PCR (qRT-PCR), after isolation of total RNA, mRNA was reverse-transcribed with SuperScript III First-Strand Synthesis System (Thermo-Fisher Scientific). qRT-PCR reactions were conducted using SsoFast EvaGreen Supermix on a CFX96 Real-Time PCR System (Bio-Rad). For RNA-seq, the barcoded libraries were prepared with Illumina RNA preparation kit according to manufacturer's instruction. The libraries were sequenced using HiSeq 2500 sequencing system (Illumina). Basic data processing and differential expression testing were performed using the DESeq2 package in R. Principal component analysis of DEGs (WT vs *Rai1*$^{STOP/+}$ and WT vs rescue) was performed in R using the prcomp function. Differentially expressed genes had $\log_2$ FC > 0.5 and FDR adjusted p

values < 0.05. Hierarchical clustering was performed using complete linkage with Euclidean distances. GO analysis was performed using the GO enrichment tool: http://geneontology.org/page/go-enrichment-analysis

We previous found that *Rai1* exons 4–6 were up-regulated when the major exon 3 was inhibited (1). As a result, *Rai1* was identified as an up-regulated gene in these conditional mutants. Our qRT-PCR experiments using primers spanning exons 3-4 junctions have confirmed that *Rai1* exon 3 was indeed down-regulated in $Rai1^{STOP/+}$ mice. Therefore, we removed *Rai1* from the up-regulated DEG list to prevent confusion. For list of differentially expressed genes identified in Rai1-deficient cortex and striatum, see Datasets 1 and 2. For qRT-PCR, after isolation of total RNA from desired brain regions, mRNA was reverse-transcribed with SuperScript III First-Strand Synthesis System (Thermo-Fisher Scientific). qRT-PCR reactions were conducted using SsoFast EvaGreen Supermix (Bio-Rad) on a CFX96 PCR System (Bio-Rad). For qRT-PCR primer sequences, see Dataset 3.

Mouse behavioral assays

Littermates with the same genotype were housed in the same cage (n=4–5/cage) and used in behavioral assays. Behavioral assays were conducted in at least 2–3 independent cohorts of mice to ensure robustness of the results. Each cohort followed the same sequence of behavioral tests as listed below. Mice were habituated to handling for 3 days prior to the onset of the first behavioral tests. At least one day were given between assays for mice to recover. Randomization of mice was performed, and experimenters were blind to mouse genotype during behavioral testing and data analysis.

Activity chamber

Mice were placed for 10 minutes in the center of a 43.2 x 43.2 cm square arena in a 66 x 55.9 x 55.9 cm sound-attenuating chamber under dim red light. Mice were allowed to move freely in the chamber, and the time spent in the center versus periphery of the arena, distance moved, average velocity, and vertical movement were determined from infrared sensors using an automated system. At the end of each trial, the surface of the arena was cleaned with 1% Virkon.

Y maze

Mice were placed in a Y-shaped maze consisting of white plastic arms positioned 120° apart radially. All arms were 12.7 cm high and 7.62 cm wide; two arms were 15.24 cm in length, and one arm was 20.32 cm in length. Each mouse was allowed to explore the maze for 5 minutes, and the order of arm entries was recorded. The sequence of arm entries was broken into overlapping, consecutive triads (total number of triads = total number of arm entries – 2). The percent alternation was defined as (number of triads containing entry into all three arms / total number of triads) X 100.

Vertical pole descent test

Mice were placed at the top of a coarse, vertical wooden pole (diameter: 1 cm; height: 55 cm), and the time required to descent was recorded. After a practice trial, 4 test trials were conducted with an inter-trial interval of ~2 minutes. If the mouse did not descend or dropped or slipped down the pole without climbing, a descent time of 60 seconds was recorded.

Sociability

Mice were placed in a testing cage containing two empty inverted cups and were allowed to habituate for 60 minutes. An unfamiliar mouse (C57BL/6 juvenile) was then placed under one of the cups, and the time spent by the subject mouse investigating the two cups was quantified for five minutes. The intruder mouse was then removed, and an inter-trial interval of 15 minutes was allotted. Following this interval the original intruder mouse was placed back under one of the cups (opposite the cage from original placement to control for test subject place preference) and a second, non-cage mate intruder mouse (C57BL/6 juvenile) was placed under the other cup. The time spent by the subject mouse investigating the two cups was quantified for five minutes, after which all mice were returned to their home cages.

Delayed Match-to-Place Water Maze

Mice were placed individually in a circular tank (diameter: 150cm) which was filled with water to a depth of approximately 45 cm. Grey tempera paint was added to the water to obscure a platform (diameter: 17cm) submerged 1 cm beneath the water's surface. The tank was surrounded on three sides by privacy blinds, each with a distinct visual cue. In each trial mice were allowed to swim for 90 seconds or until they remained on the hidden platform for three seconds (maze escape). If the mouse did not find the platform within the allotted 90 seconds, it was guided there by an experimenter and allowed to remain on the platform for 10 seconds. Mice were tested a total of four times each day. Mice were introduced into the maze in semi-randomized locations each trial and the platform was moved to a new location at the beginning of each testing day. All trials were monitored by an overhead camera and Ethovision monitoring software and analyzed for variables including escape latency, distance moved, thigmotaxis

(swimming along edge), and average velocity. Savings T1-T2 was calculated by the average of trial 1 minus the average of trial 2 for each animal. Saving T1-T4 was calculated by the average of trial 1 minus average of trial 3 for each animal.

Fear conditioning

On Day 1 (training), mice were placed in Context A and after 3 minutes, they were presented with a tone (75 dB, 2 kHz, 20 sec) followed 18 seconds later by a footshock (0.5 mA, 2 seconds); mice received a total of five tone-shock pairings with an inter-tone interval (from the end of one tone to the start of the next tone) of 80 seconds. On Day 2 (contextual recall), mice were placed in Context A for 5 minutes without any tone presentation. On Day 3 (cued recall) mice were placed for 3 minutes in Context B, which had different olfactory, somatosensory, and visual cues from Context A. They were subsequently presented with three 20s tone presentations (80 seconds inter-tone interval) without any shocks. Freezing, defined as complete lack of motion for at least 0.75 seconds, was quantified on all three days by an automated video scoring system (FreezeFrame, Actimetrics).

Hot plate assay

Mice were placed on a hot plate (Model 39, IITC) heated to either 50°C or 55°C, and the latency to exhibit pain behavior (paw lifting, paw licking, or jumping) was recorded. Mice were tested in three trials per temperature. Hot plate experiments were conducted during the subjective day rather than the subjective night.

Optogenetic photostimulation in the tube test

AAVs containing *hSyn-hChR2(H134R)-EYFP* and *hSyn-EYFP* were purchased from UNC vector core. 1 µl AAV2 was unilaterally injected into the right side of mPFC (AP: +2.43, ML: +0.4, DV: –1.6 mm from Bregma). Mono fiber-optic cannulae were implanted on the same day during viral injection, 200 µm above viral injection coordinates. Virus was allowed to express for at least a month before photostimulation. Mice implanted with optic connectors were then trained to walk through the tube (30 cm in length, 3 cm in diameter, with a 12 mm slit opened at the top) for 10 times with fiber connected each day for two days. Computerized randomization was used to ensure that each mouse only encountered a novel opponent twice (once during laser-off and once during laser-on condition), and the experimenters were blind to the genotype of the mice and the injected virus. On the laser-off test day, pairs of mice were released at the two ends of a tube and met at the middle. The mouse that retreated first from the tube was designated as the loser. The tube test was video recorded and total numbers of winning were calculated for each genotype/virus injected condition. One week after the laser-off test, the same procedure were conducted except a 473 nm blue light (30 mW) was turned on right before mice entering the tube. The matches were also randomly assigned, video recorded, and the winning percentage induced by light stimulation was calculated. Four mice from each genotype/virus injected condition were used for each cohort (n=12 for each cohort), and three independent cohorts of mice were tested. For a cohort of 12 mice, all 48 possible pairs of mice were tested over two testing days. The order of trials was randomly generated with the condition that individual mice were allowed at least 5 minutes between trials.

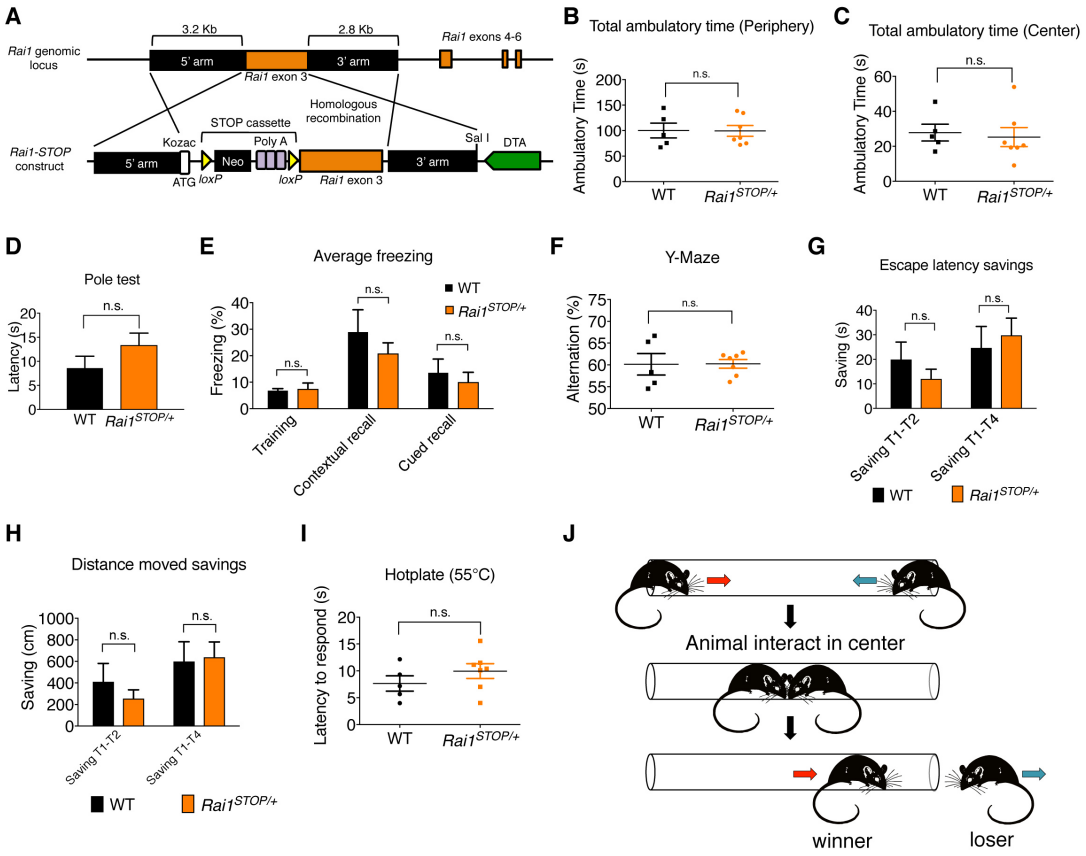

Fig. S1. Neurobehavioral characterization of male *Rai1*$^{STOP/+}$ mice

A. Schematic representation of *Rai1*$^{STOP/+}$ mouse design.
B. Time spent in the periphery of the activity chamber was not significantly different between WT and *Rai1*$^{STOP/+}$ mice (n.s., not significant; t-test).
C. Time spent in the center of the activity chamber was not significantly different between WT and *Rai1*$^{STOP/+}$ mice (t-test).
D. Motor function evaluated by pole test showing a similar latency for the *Rai1*$^{STOP/+}$ (n=7) mice to climb down the pole as WT (n=5) mice (t-test).
E. The performance of *Rai1*$^{STOP/+}$ mice (n=7) in fear conditioning test was indistinguishable from the WT mice (n=5), suggesting normal fear learning and memory (t-test).
F. *Rai1*$^{STOP/+}$ mice showed similar performance in the Y-maze as WT mice, suggesting normal spatial memory (t-test).
G. *Rai1*$^{STOP/+}$ mice (n=7) showed similar escape latency savings (quantified by the difference in escape latency during the first trial and remaining trials) to WT mice (n=5) in the Morris mater maze, suggesting normal spatial learning and memory function (t-test).
H. *Rai1*$^{STOP/+}$ mice (n=7) showed similar distance moved savings to WT mice (n=5) in the Morris mater maze, suggesting normal spatial learning and memory function (t-test).
I. Pain sensitivity remained normal in the *Rai1*$^{STOP/+}$ mice as shown by the hot plate test (t-test).
J. Schematic showing the procedure of a tube test. The loser mouse either retreated voluntarily or was pushed out. Either way, the mouse stayed in the tube was declared the "winner".

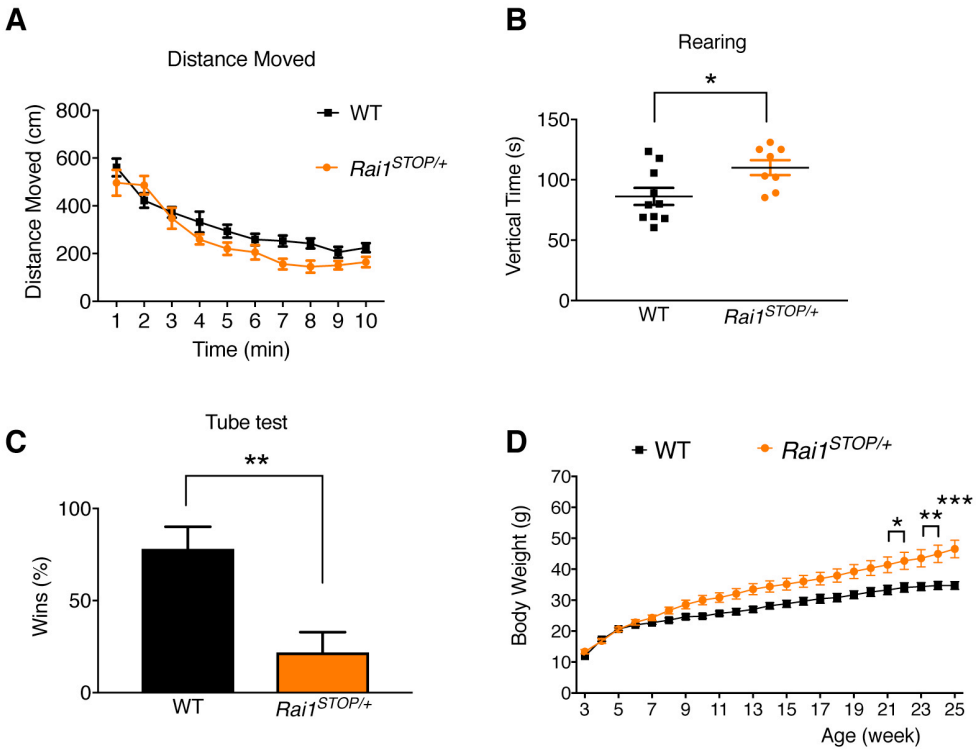

Fig. S2. Neurobehavioral characterization of female *Rai1*$^{STOP/+}$ mice

A. Locomotor activity was indistinguishable between WT (n=10) and *Rai1*$^{STOP/+}$ mice (n=8).
B. *Rai1*$^{STOP/+}$ mice showed increased rearing activity (*p<0.05; t-test).
C. *Rai1*$^{STOP/+}$ mice showed abnormal social interaction in the tube test (**p<0.01; t-test).
D. Comparison of the body weight of WT (n=10) and *Rai1*$^{STOP/+}$ (n=8) mice (mean±S.E.M.; *p<0.05, **p<0.01, ***p<0.001; two-way ANOVA followed by Bonferroni post-hoc test).

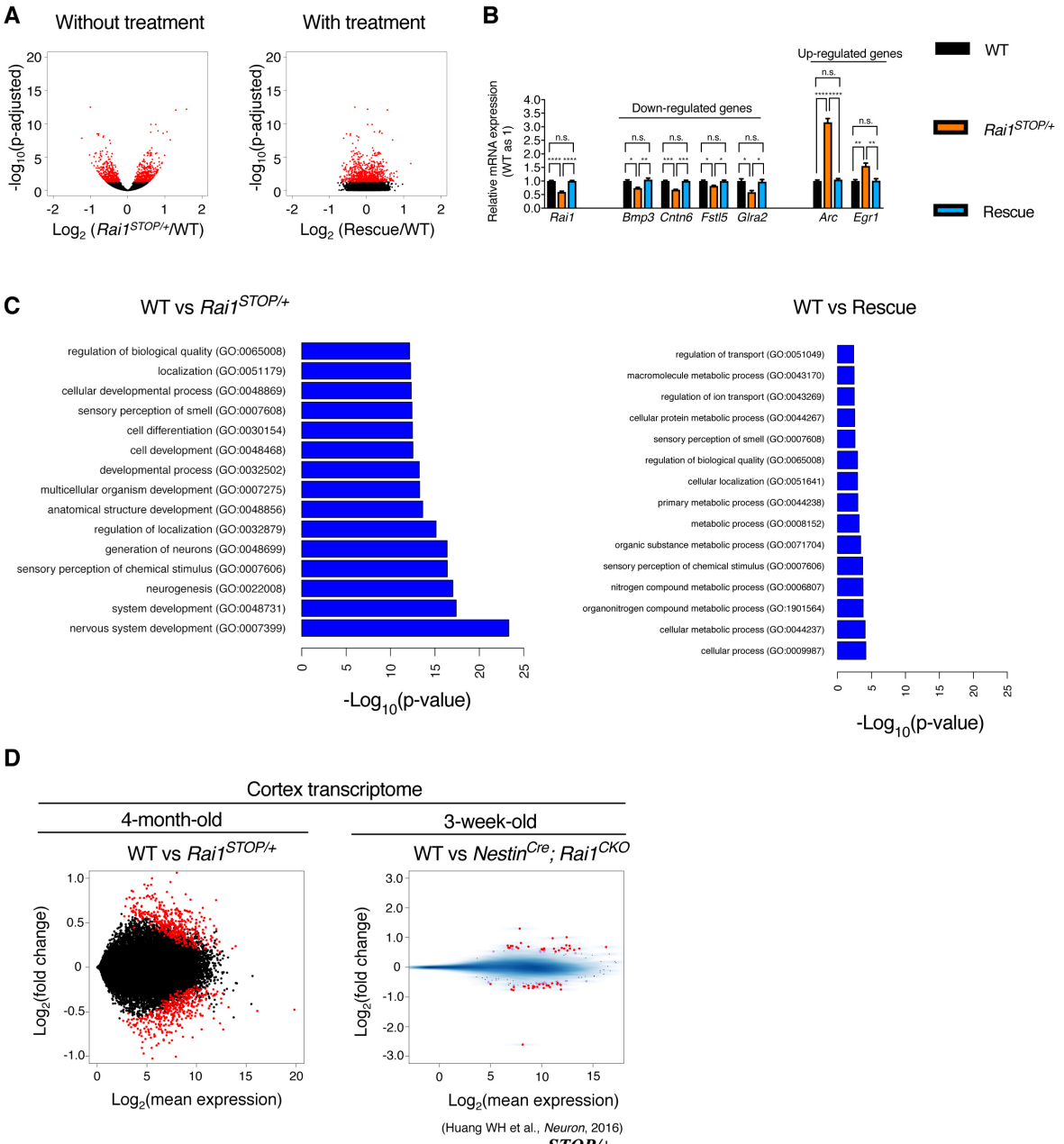

Fig. S3. Transcriptomic analysis of WT, *Rai1*$^{STOP/+}$, and rescue mice

A. Volcano plot of RNA-seq results showing that *Rai1* reactivation (treatment) decreased the number of differentially expressed genes (DEGs) and transcriptional fold change. Red dots show genes with False Discovery Rate (FDR) < 0.05.

B. *Rai1* reactivation normalized selective down- and up-regulated genes in the *Rai1*$^{STOP/+}$ brain as validated by qRT-PCR (n=3 samples; n.s., not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; one-way ANOVA followed by Tukey's post-hoc test).

C. Gene ontology analysis using DEGs obtained from WT vs *Rai1*$^{STOP/+}$ cortex (left) and WT vs rescue cortex (right).

D. Genome-wide gene expression changes in 4-month-old *Rai1*$^{STOP/+}$ cortex (left) and 3-week-old *Nestin*^{Cre};*Rai1*^{CKO} cortex from our previous study (right) compared to corresponding control cortices. Red dots show genes with False Discovery Rate < 0.05.

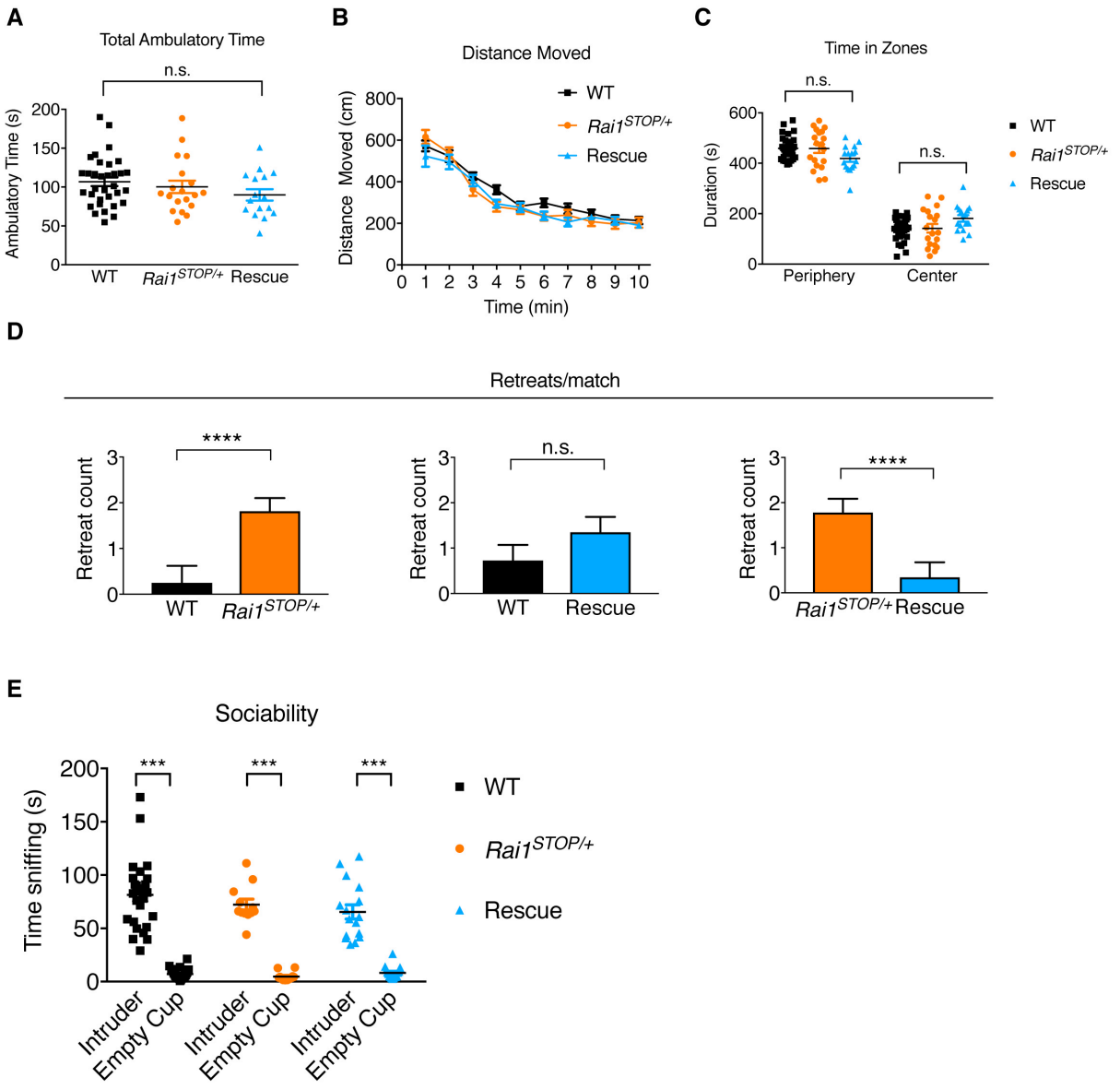

Fig. S4. Neurobehavioral characterization of juvenile and adult rescue mice

A. Total ambulatory time in the activity chamber was not different between WT (n=33), *Rai1*$^{STOP/+}$ (n=19), and juvenile rescue mice (n=16) (n.s., not significant; one-way ANOVA).

B. Distance moved in the activity chamber was not different between WT (n=33), *Rai1*$^{STOP/+}$ (n=19), and juvenile rescue mice (n=16).

C. Time spent in the periphery and center of the activity chamber was not different between WT (n=33), *Rai1*$^{STOP/+}$ (n=19), and juvenile rescue mice (n=16) (one-way ANOVA).

D. Video recording followed by quantification (blind to genotype) showing that *Rai1*$^{STOP/+}$ mice (n=32) showed increased retreat frequency when encountering both WT (n=32) and juvenile rescue (n=37) mice (****p<0.0001; t-test).

E. Home cage sociability test showing that WT, *Rai1*$^{STOP/+}$, and juvenile rescue mice all preferred novel intruder over an empty cup, suggesting normal social motivation (***p<0.001; t-test).

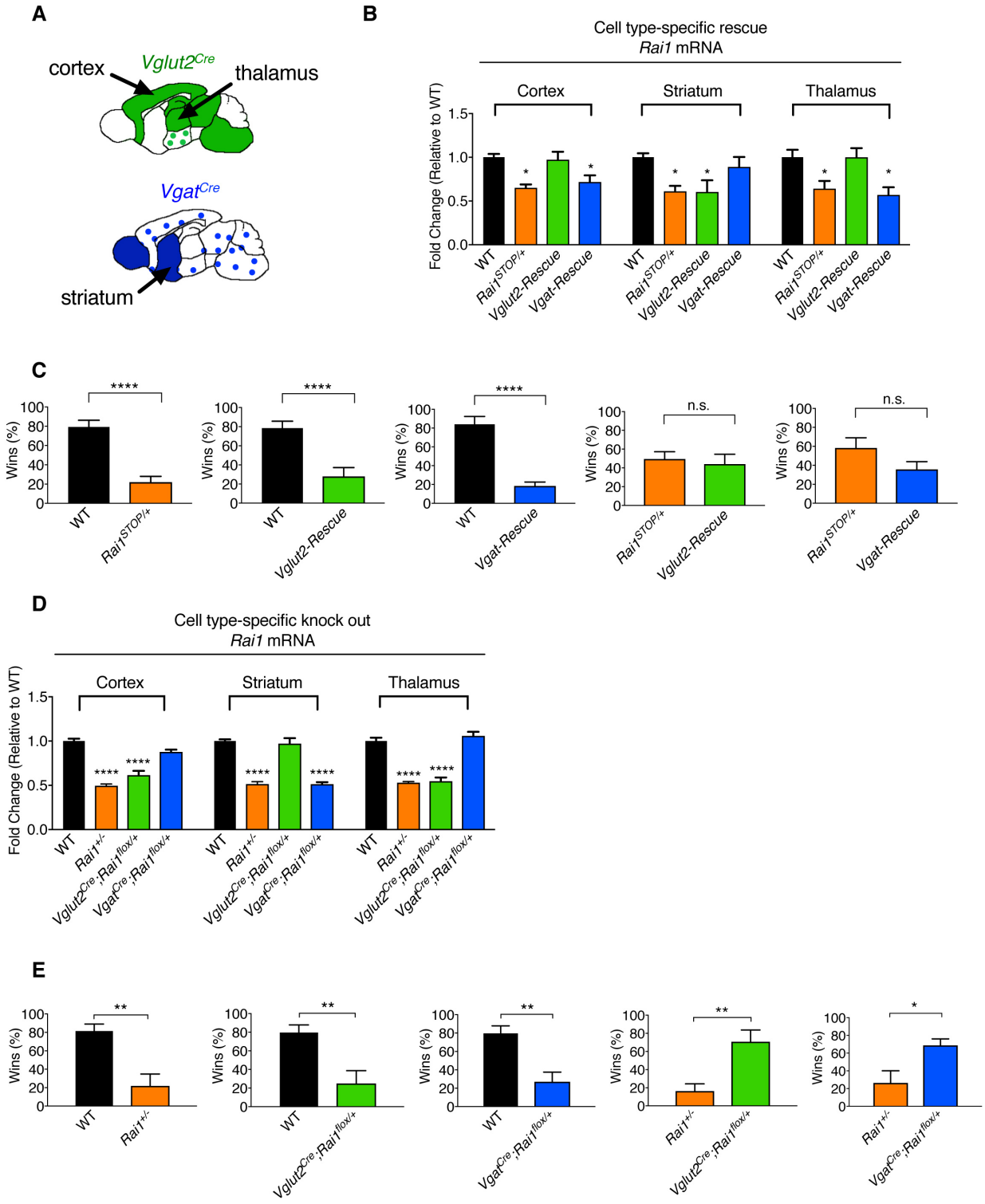

Fig. S5. Cell type-specific rescue and deletion of Rai1

A. Schematic showing the brain regions targeted by cell type-specific Cre lines. Note that although *Vglut2*^{Cre} is expressed in subcortical but not cortical excitatory neurons in adult, it is expressed transiently in cortical excitatory neurons during development, such that Cre-mediated reactivation of *Rai1* in the *Rai1*^{STOP} allele or Cre-mediated deletion of *Rai1*

in the *Rai1*^{flox} allele occurs efficiently in cortical excitatory neurons as well (see panels B and D).

B. Quantitative RT-PCR showing that *Rai1* was reactivated in brain regions enriched with Cre-expressing cells. Cortex contain ~80% of excitatory and ~20% of inhibitory neurons. Striatum and thalamus are highly enriched with inhibitory and excitatory neurons, respectively (n=3 samples; *p<0.05; one-way ANOVA followed by Tukey's post-hoc test; each genotype was compared to WT).

C. *Rai1*$^{STOP/+}$ (n=17), *Vglut2*-Rescue (n=14), and *Vgat*-Rescue (n=14) mice showed abnormal social interaction in the tube test when encountering WT mice (n=15). In contrast, *Vglut2*-Rescue, and *Vgat*-Rescue mice showed similar social interaction in the tube test when encountering *Rai1*$^{STOP/+}$ mice (n.s., not significant, ****p<0.0001; t-test).

D. Quantitative RT-PCR showing that one copy of *Rai1* was deleted in brain regions enriched with Cre-expressing cells and all brain regions of *Rai1*$^{+/-}$ mice (n=3 samples; ****p<0.0001; one-way ANOVA followed by Tukey's post-hoc test; each genotype was compared to WT).

E. *Vglut2*^{Cre};*Rai1*$^{flox/+}$ (n=10) and *Vgat*^{Cre};*Rai1*$^{flox/+}$ (n=10) mice showed abnormal social interaction when encountering unfamiliar WT mice (n=10); however, both *Vglut2*^{Cre};*Rai1*$^{flox/+}$ and *Vgat*^{Cre};*Rai1*$^{flox/+}$ mice won over *Rai1*$^{+/-}$ mice (n=10) (*p<0.05, **p<0.01; t-test).

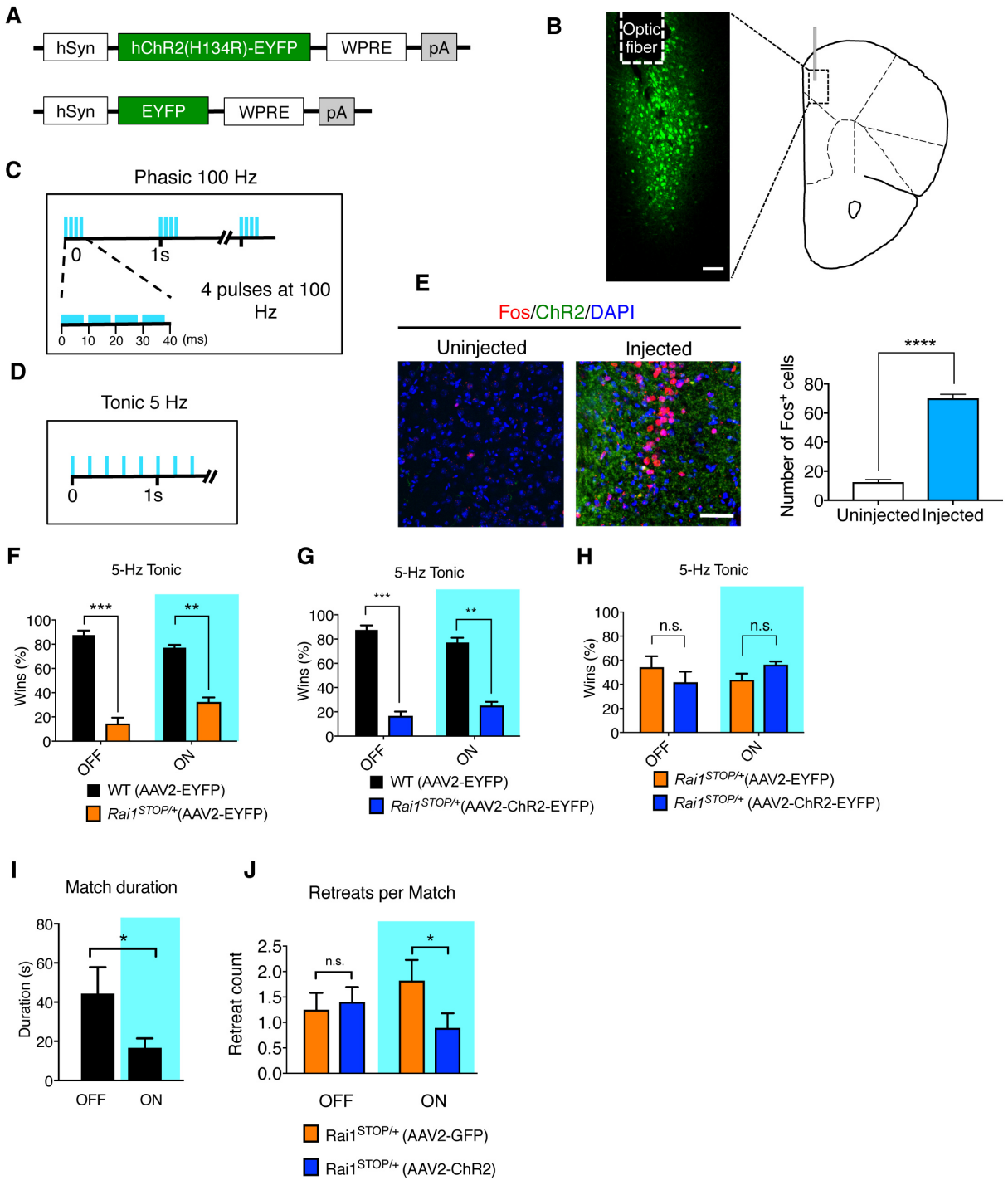

Fig. S6. Phasic but not tonic activation of PFC partially rescued social interaction deficit of *Rai1*$^{STOP/+}$ mice

A. AAV constructs for optogenetic activation and control used in this study. hSyn, human synapsin promoter; WPRE, woodchuck hepatitis virus regulatory element ; pA, polyA.

B. Left: Coronal section of mPFC showing viral injection and optic fiber implantation sites. Scale bar: 100 μm. Right: fiber placement in mPFC for optogenetic stimulation.

C. Schematic for the phasic 100-Hz light stimulation protocol (100-Hz phasic, 5 ms per pulse, four pulses per second).
D. Schematic for the tonic 5-Hz (15 ms per pulse) light stimulation protocol.
E. Top: ChR2-EYFP (green) expressing PFC neurons were activated with 100-Hz photostimulation and co-stained with Fos (red) and DAPI (blue). Bottom: Fos quantification in ChR2-EYFP uninjected and injected sides of PFC (n=3 for each condition; mean±S.E.M.; ****p<0.0001; t-test).
F. Tonic photostimulation did not affect social interaction of WT-EYFP (n=12) and *Rai1*$^{STOP/+}$-EYFP (n=12) mice (mean±S.E.M.; **p<0.01, ***p<0.001; t-test).
G. Tonic photostimulation did not affect social interaction of WT-EYFP (n=12) and *Rai1*$^{STOP/+}$-ChR2-EYFP (n=12) mice (mean±S.E.M.; **p<0.01, ***p<0.001; t-test).
H. Tonic photostimulation did not affect social interaction of *Rai1*$^{STOP/+}$-ChR2-EYFP (n=12) when encountering *Rai1*$^{STOP/+}$-EYFP (n=12) mice in the tube test (mean±S.E.M.; n.s., not statistically different; t-test).
I. Match duration between *Rai1*$^{STOP/+}$-ChR2-EYFP (n=25) and *Rai1*$^{STOP/+}$-EYFP (n=29) mice was reduced upon mPFC activation (mean±S.E.M.; *p<0.05; t-test).
J. *Rai1*$^{STOP/+}$-ChR2-EYFP (n=29) mice showed a decreased retreat count from social encounters in the tube test upon mPFC activation when compared to *Rai1*$^{STOP/+}$-ChR2-EYFP (n=29) mice (mean±S.E.M.; n.s., not statistically different, *p<0.05; t-test).

Reference

1. Huang WH*, et al.* (2016) Molecular and Neural Functions of Rai1, the Causal Gene for Smith-Magenis Syndrome. *Neuron* 92:392-406.