Molecular and Neural Functions of Rai1, the Causal Gene for Smith-Magenis Syndrome

Highlights

- Rai1 preferentially binds active promoters and promotes transcription
- Pan-neural loss of Rai1 causes motor function and learning deficits and obesity
- Rai1 loss in inhibitory and subcortical excitatory neurons causes learning deficits
- Rai1 loss in subcortical excitatory, Sim1+, and SF1+ neurons causes obesity

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In Brief

Huang et al. show that Rai1, the causal gene for neurodevelopmental disorder Smith-Magenis syndrome (SMS), binds active promoters and promotes transcription. Rai1 loss in different neuronal subtypes gives rise to specific SMS-like deficits in motor function, learning, and food intake.
Molecular and Neural Functions of Rai1, the Causal Gene for Smith-Magenis Syndrome

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SUMMARY

Haploinsufficiency of Retinoic Acid Induced 1 (RAI1) causes Smith-Magenis syndrome (SMS), which is associated with diverse neurodevelopmental and behavioral symptoms as well as obesity. RAI1 encodes a nuclear protein but little is known about its molecular function or the cell types responsible for SMS symptoms. Using genetically engineered mice, we found that Rai1 preferentially occupies DNA regions near active promoters and promotes the expression of a group of genes involved in circuit assembly and neuronal communication. Behavioral analyses demonstrated that pan-neural loss of Rai1 causes deficits in motor function, learning, and food intake. These SMS-like phenotypes are produced by loss of Rai1 function in distinct neuronal types: Rai1 loss in inhibitory neurons or subcortical glutamatergic neurons causes learning deficits, while Rai1 loss in Sim1* or SF1* cells causes obesity. By integrating molecular and organisational analyses, our study suggests potential therapeutic avenues for a complex neurodevelopmental disorder.

INTRODUCTION

Copy number variations (CNVs) cause numerous neurodevelopmental and psychiatric disorders (Malhotra and Sebat, 2012; Ramocki and Zoghbi, 2008). It is generally difficult to dissect the etiology and develop effective treatments for disorders associated with large CNVs due to uncertainty about which genes within a deletion or duplication are responsible for the symptoms (Zhang et al., 2009). A prominent CNV disorder is Smith-Magenis syndrome (SMS) (Smith et al., 1993), which affects one in 15,000 individuals. SMS patients exhibit craniofacial abnormalities, obesity, circadian abnormality, hypotonia, intellectual disabilities, stereotypies, and autistic features (Greenberg et al., 1996; Smith et al., 1993). 70% of SMS patients have an ~3.7 Mb interstitial deletion of chromosome 17p11.2 that contains 76 genes (Elsea and Girirajan, 2008). Importantly, 10% of SMS patients harbor point mutations or small deletions causing haploinsufficiency of a single gene within this region, Retinoic Acid Induced 1 (RAI1) (Dubourg et al., 2014; Slager et al., 2003). Patients with RAI1 mutations exhibit almost all of the core features of SMS, indicating that RAI1 is the dosage-sensitive gene responsible for most symptoms even in patients with large deletions. Furthermore, the reciprocal duplication in 17p11.2 causes Potocki-Lupski syndrome (PTLS), which shares many neuropsychological symptoms with SMS (Potocki et al., 2000, 2007). The smallest region common to PTLS patients with different duplications is a 125-kb region containing only RAI1 (Zhang et al., 2010), suggesting that duplication of RAI1 may also be responsible for the symptoms of PTLS. Therefore, brain development and function is exquisitely sensitive to RAI1 copy number.

RAI1 is a nuclear protein with two predicted protein-interacting domains: an extended plant homeo-domain (ePHD) and a nucleosome-binding domain (NBD) (Darvekar et al., 2012, 2013). In vitro studies revealed that overexpressed RAI1 interacts with nuclear structures with high affinity (Darvekar et al., 2012). Furthermore, the RAI1 NBD can interact with HeLa nucleosomes (Darvekar et al., 2013). Overexpressed RAI1 binds to Brain-Derived Growth Factor (BDNF) and Circadian Locomotor Output Cycles Kaput (CLOCK) enhancers in vitro (Burns et al., 2010; Williams et al., 2012). While RAI1 does not possess a known DNA binding domain, when fused with a GAL4 DNA-binding domain, RAI1 shows moderate transcriptional activity in a luciferase assay (Bi et al., 2005). Although these data suggest a role for RAI1 in transcriptional regulation, its in vivo mode of action and targets remain unknown.

Mouse models have been used to study Rai1 function in vivo. Using an Rai1LacZ/+ allele that expresses β-galactosidase from the mouse Rai1 locus, it was found that Rai1 is expressed in many tissues including the developing and adult nervous system (Bi et al., 2005). Most Rai1 null mice die in utero; the few that survive exhibit craniofacial and skeletal abnormalities, motor

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dysfunction, and fear-learning deficits (Bi et al., 2007). Rai1 heterozygous mice display mild SMS-like symptoms including obesity, circadian abnormalities, and characteristic craniofacial features (Bi et al., 2005; Lacaria et al., 2013). Overexpression of Rai1 in mice results in growth retardation, hyperactivity, and motor deficits (Girirajan et al., 2008). Given the broad expression pattern of Rai1 and diverse SMS symptoms, it is critical to determine whether specific symptoms are results of Rai1 requirement in specific cell types, in order to understand SMS pathogenesis and develop targeted therapies. Furthermore, while removing one copy of Rai1 better mimics the genetic underpinnings of SMS, deleting both copies in specific cell types may result in more severe phenotypes that reveal biological functions of Rai1.

Here, we have taken an integrative approach to dissect the molecular and neural functions of Rai1. Using a Rai1 conditional allele and an epitope-tagged Rai1 allele, we found that Rai1 preferentially occupies DNA regions near active promoters and enhances the expression of genes that function in cell-cell communication. We identified cell types that require Rai1 for proper motor function, learning, and food intake. Our integrative approach provides mechanistic insights into the etiology of SMS and suggests specific therapeutic strategies.

RESULTS

Rai1 Is Broadly Expressed in Postmitotic Neurons

To characterize Rai1 expression and to analyze its molecular functions, we engineered knockin mice with tandem FLAG and myc peptides (hereafter, Tag) fused to the carboxyl terminus of endogenous Rai1 (Rai1-Tag; Figure 1A). Western blot showed that the anti-FLAG antibody specifically recognized Rai1-Tag but not endogenous Rai1 (Figure S1A). Rai1-Tag was expressed...
at a similar level as un-tagged Rai1 (Figure S1B), as shown by an anti-Rai1 antibody we developed (and validated by lack of staining in conditional knockout; see below). Immunostaining revealed that Rai1-Tag co-localized with signals detected by the anti-Rai1 antibody in the postnatal day (P) 21 somatosensory cortex (white arrowheads, Figure 1B). 99.6% Rai1+ cells expressed Tag, 98.2% of the Tag+ cells expressed Rai1 (Figure 1B; quantified from 1,432 DAPI+ cells from 9 sections). We conclude that Rai1-Tag faithfully represents the endogenous Rai1 expression and used Rai1-Tag to further characterize Rai1 expression.

Consistent with a previous report (Bi et al., 2005), we observed Rai1-Tag expression in the embryonic day 9.5 (E9.5) branchial arch (Figure S1C) that develops into craniofacial structures. In E18.5 cortex, Rai1-Tag was broadly expressed in the cortical plate enriched in postmitotic neurons but rarely in the Ki67+ actively dividing cells near the ventricular zone (Figure 1C). The enrichment of Rai1 in postmitotic but not in proliferating cells was also observed in the developing diencephalon (Figure S1D and Table S1), cerebellum, and olfactory cortex (data not shown). Rai1 was also detected in a small fraction of S100β+ cerebellar Bergmann glia (Figure S1E and Table S1). Consistent with immunostaining, qRT-PCR using mouse cortices indicated that Rai1 mRNA levels increased during prenatal development, peaked around 1 week after birth, and persisted into adulthood (Figure 1D). Rai1-Tag was broadly expressed throughout the adult mouse brain (Figure 1E) and co-localized with 78% of the NeuN+ cortical neurons (Figure S1F and Table S1). Rai1 is expressed in both excitatory and inhibitory neurons in the thalamus (Figures S1G–S1J) and cortex (Figures S1K and S1L). Quantification based on double labeling of Rai1-Tag and in situ hybridization showed that Rai1 is expressed in 75% of excitatory neurons expressing Vglut1 (encoding vesicular glutamate transporter 1) and 57% of inhibitory neurons expressing Gad1 and/or Gad2 (encoding glutamate decarboxylases) in cortex (Figures S1K and S1L and Table S1). In summary, Rai1 is expressed in many cell types in the brain, with an onset that parallels the neuronal differentiation process.

**Rai1 Occupies DNA Regions Near Active Promoters In Vivo**

Mouse Rai1 and human RAII share the same protein structure, with 82% overall sequence identity, and 88% and 82% identity in the C-terminal NBD and ePHD, respectively (Figure 2A). Human RAII[1] was shown to interact with nucleosomes in vitro (Darvekar et al., 2013). To further characterize molecular functions of Rai1, we purified the recombinant human and mouse NBDs and ePHDs expressed in *E. coli* and performed a nucleosome pull-down assay. We found that NBDs from both species interact with HeLa nucleosomes, whereas an equal amount of ePHD protein did not (Figures 2B, S2A, and S2B). In a cellular fractionation assay using mouse cortices, Rai1 was present in both nucleoplasmic and chromatin-binding fractions (Figure 2C). These experiments suggest that Rai1 interacts with chromatin in vitro and in vivo.

To investigate the genome-wide DNA binding pattern of Rai1 in vivo, we performed chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) using Rai1[1] mice. After confirming that FLAG-tagged Rai1 was enriched in anti-FLAG immunoprecipitation (Figure S2C), we performed ChIP-seq using 8-week-old Rai1[1]/+ mouse cortices. We obtained ~35 million paired-end reads, which exhibited a bimodal enrichment pattern along the mouse genome for each of the two replicates (Figure S2D and Table S2). We identified ~15,000 reproducible peaks (irreproducible Discovery Rate < 0.05) as high-confidence Rai1 binding sites (Figure S2E and Table S3). ChIP-qPCR also validated Rai1 binding sites identified by ChIP-seq (Figure S2F). Genome-wide enrichment analysis revealed that Rai1 binding sites were enriched in CpG islands, 5′ UTRs, and promoters (Figure 2D), but not intergenic or repetitive regions (Figure S2G). Normalized Rai1 ChIP-seq read densities on RefSeq gene bodies showed strong enrichment around transcriptional start sites (TSSs) (Figure 2E). These results demonstrate that Rai1 preferentially occupies promoter regions. We then categorized regions of the mouse genome into different states based on combinations of active or repressive chromatin marks (Ernst and Kellis, 2012). The overlay of Rai1 binding sites with different chromatin states further indicated that Rai1 binds active promoter and enhancer regions (Figures 2F and S2H; Table S4).

To determine the specificity of Rai1 binding to different DNA sequences, we applied a de novo motif-discovery algorithm (Heinz et al., 2010) on the high-confidence Rai1 binding sites. Among the significantly over-represented motifs (Figure S2I), an 8-mer motif was found in 45% Rai1 binding sites (Figure 2G; p = 1e–58). A comparison with previously discovered DNA motifs revealed that the 8-mer motif resembles the consensus sequences bound by zinc finger transcription factors ZNF711 and Zfx (Figure 2G). Interestingly, ZNF711 is highly expressed in the brain (Kleine-Kohlbrecher et al., 2010), and truncating mutations of ZNF711 have been identified in X-linked mental retardation (Tarpey et al., 2009). It remains to be experimentally determined whether these similar binding motifs are a consequence of physical interactions between Rai1 and ZNF711/Zfx, or could result in their competition. Comparisons of the positions between Rai1 peaks with existing mouse Zfx ChIP-seq data (Chen et al., 2008) revealed that Zfx binding signals and Rai1 peaks were globally co-localized, along with RNA polymerase 2 (Pol2) binding sites and the permissive transcription mark H3K4me3 (Figure 2H). By ranking the ChIP-seq peaks based on Rai1 peak intensity, we generated a heatmap showing the corresponding ChIP-seq read intensities of H3K4me3, Pol2, Zfx, and a negative control (anti-GFP antibody) (Figure 2I). The heatmap showed a high co-occupancy between Rai1 peaks with H3K4me3, Pol2, and Zfx peaks, but not with the negative control. Together, our genome-wide analysis indicates that Rai1 binds to specific loci in the genome associated with active transcription.

**Rai1 Positively Regulates Steady-State Transcription**

Given that Rai1 binds to promoter regions, we next examined how loss of Rai1 impacts the transcriptome. To circumvent embryonic lethality of Rai1 null mice, we generated a conditional knockout (CKO) allele by flanking exon 3, which encodes 97% of the Rai1 open reading frame including the translational start, with loxP sites (Figures S3A and S3B). Rai1[loxP/loxP] mice were born at the expected Mendelian ratio without apparent abnormalities. We then conditionally deleted Rai1 in the nervous
Figure 2. Rai1 Preferentially Occupies the Promoter Regions of Active Chromatin
(A) Schematic representation of predicted Rai1 protein domains. The nucleosome binding domain (NBD, blue) and extended plant homeo-domain (ePHD, brown) of human RA1 and mouse Rai1 show a high degree of sequence conservation (identical amino acids are indicated as vertical green lines at bottom). In the magnified C termini of both proteins, numbers indicate amino acids in the primary sequence, and thin lines indicate gaps in alignment.
(B) In vitro nucleosome interaction assay showing that both human and mouse NBDs, but not ePHDs, bind purified HeLa nucleosomes.
(C) Cellular fractionation assay using mouse cortex showing that endogenous Rai1 co-fractionated with both histone H3 (chromatin fraction) and HDAC2 (nucleoplasmic fraction).
(D) Genome-wide annotation showing Rai1 binding sites identified by ChIP-seq are highly enriched at CpG island, 5' UTR, and promoter regions. ncRNA, non-coding RNA; TES, transcription end site.

(legend continued on next page)
Figure 3. Transcriptomic Changes in Rai1 Mutant Brains

(A) UCSC browser tracks of the cortex RNA-seq data of the Rai1 locus. The expression of floxed exon 3 (red box) is largely absent in the Nestin<sup>Cre⁺⁺</sup>;Rai1<sup>CKO⁻⁻</sup> cortex, while exons 4 to 6 are all upregulated compared to control. Coding exons, tall boxes.

(B) qRT-PCR showing Cre-dependent decrease of Rai1 exon 3 mRNA expression, and upregulation of Rai1 exons 4/5. Data are mean ± SEM (*p < 0.05, unpaired t test, n = 3).

(C) Genome-wide gene expression change in 3-week-old Nestin<sup>Cre⁺⁺</sup>;Rai1<sup>CKO⁻⁻</sup> cortex compared to control. Red dots show genes with False Discovery Rate (FDR) < 0.05 and brown dots show genes with 0.05 < FDR < 0.1.

(D) Genes with Rai1 binding sites identified by ChIP-seq in cortex showing a leftward shift in the cumulative distribution plot (red) compared with cumulative distribution of all genes (black), suggesting a global downregulation of Rai1-bound genes in the Rai1 mutant cortex.

(E) Downregulated genes in Gad2<sup>Cre⁺⁺</sup>;Rai1<sup>CKO⁻⁻</sup> striatum at 3 weeks are still downregulated at 12 weeks (red dots in the bottom left but not top left quadrants). However, several 3-week upregulated genes become significantly downregulated at 12-weeks (red dots in the bottom right quadrant).

(F) Gene ontology analysis of downregulated genes in cortex and striatum showing enrichment in cell adhesion and morphogenesis-related terms. Gene ontology terms are ranked by FDR.

See also Figure S3.
enhanced splicing efficiency after Cre-mediated deletion of the preceding exon 3. Globally, RNA-seq showed that loss of Rai1 caused changes in the expression levels of a small number of genes (Figure 3C), a subset of which were independently verified with qRT-PCR (Figure S3D). To further understand how Rai1 regulates transcription, we combined the ChIP-seq and RNA-seq data obtained from the cortex. We found that Rai1-bound genes were globally downregulated in the NestinCre;Rai1CKO brains; the fold changes were small but highly significant (Figure 3D). Therefore, Rai1 appears to positively regulate expression of its direct targets. Genes upregulated in NestinCre;Rai1CKO cortices are likely due to indirect effect of Rai1 loss.

We further investigated how Rai1 regulates transcription across developmental stages. The transcriptome of young mice may more closely reflect primary transcriptional changes due to loss of Rai1, whereas the adult tissue reflects a transcriptional state after prolonged Rai1 dysfunction. We chose GABAergic neurons for this comparison as GABAergic spiny projection neurons represent 95% of all neurons within dorsal striatum, allowing neurons for this comparison as GABAergic spiny projection neurons represent 95% of all neurons within dorsal striatum, allowing us to compare Rai1 in a relatively homogeneous population. qRT-PCR showed that Rai1 exon 3 mRNA level in the Gad2Cre;Rai1CKO dorsal striatum decreased by 80% in young (3-week-old) mice and 93% in adult (12-week-old) mice compared to sex- and age-matched control littermates (Figure S3E). We performed RNA-seq using dorsal striatum collected from 3- and 12-week-old control and Gad2Cre;Rai1CKO mice. We found that most of the downregulated genes in the young mice remained downregulated in the adult stage, whereas many upregulated genes in the young mouse became downregulated in the adult stage (Figure 3E). We also confirmed these expression patterns at both time points using qRT-PCR (Figures S3E and S3F). Therefore, genes are progressively downregulated in the Rai1 mutant brain with age progression, supporting a general role for Rai1 in positively regulating steady-state gene expression.

To gain insight into the biological processes regulated by Rai1, we compiled the downregulated genes identified from cortex and striatum (both young and adult) RNA-seq data (Table S5) and performed a gene ontology enrichment test. Functional annotation showed that Rai1 regulates genes involved in cell adhesion, axon guidance, and neuronal morphogenesis (Figure 3F and Table S6), such as Cdh7, Cdh8, Cdh9, EphA7, Pcdh20 (Figure S3G, left), and Sema3a (Figure S3G, right), suggesting that Rai1 may help assemble and maintain neural circuits.

**Pan-neural Knockout of Rai1 Leads to Severe SMS-like Phenotypes**

To explore the neural functions of Rai1, we next performed a battery of quantitative behavioral assays using the NestinCre;Rai1CKO mice and their control littermates. It should be noted that although NestinCre is expressed in progenitors that give rise to most or all neurons and glia in the CNS, it is also expressed in other organs (Harno et al., 2013; Tronche et al., 1999). The birth rate of NestinCre;Rai1CKO mice conformed to a Mendelian ratio, suggesting that the embryonic lethality exhibited by Rai1 null mice originates from NestinCre-negative tissues. Due to occasional germline activity of NestinCre, we also included mice produced in our breeding regime that lacked one Rai1 allele in their entire body (Rai1<sup>+/−</sup> or Rai1<sup>+/floxed</sup>), these are the closest mouse models of SMS.

NestinCre;Rai1CKO mice were smaller than littermates prior to weaning and showed prominent hindlimb clasping (Figure S4A). More than 80% of NestinCre;Rai1CKO mice died before 25 weeks of age (Figure 4A). Most NestinCre;Rai1CKO mice that died prior to 10 weeks of age were gaunt, exhibiting prominent kyphosis and demonstrated weight loss in the 2 weeks preceding death (Figure S4B). It has previously been reported that both SMS patients and Rai1<sup>−/−</sup> mice are obese (Burns et al., 2010; Lacaria et al., 2012) and that female patients are more likely to exhibit food-seeking behavior than males (Edelman et al., 2007). In our experiments, only female NestinCre;Rai1CKO mice became significantly overweight beginning at 5 weeks of age, while male mice were not affected (Figure 4B). The cause for the sexually dimorphic phenotype is unclear, and the interpretation could be complicated by weight loss prior to lethality in some mice. At 20 weeks of age, female NestinCre;Rai1<sup>flox</sup>/− and NestinCre;Rai1CKO mice became 35% and 101% heavier than control mice, respectively (Figure 4B). We thus used male NestinCre;Rai1CKO mice for behavioral studies, so that behavioral assays would not be confounded by different body weights (Figure S4C).

First, we tested the motor function of NestinCre;Rai1CKO mice. These mice displayed normal gait in the Catwalk assay (Figures S4D and S4E). In the activity chamber, the distance (Figures 4C and S4F) and zones (Figure S4G) traveled by NestinCre;Rai1CKO mice were statistically indistinguishable from controls. The mean velocity and vertical activity (Figures S4H and S4I) traveled by NestinCre;Rai1CKO mice was also normal. In the pole test, NestinCre;Rai1CKO mice fell, slipped from the pole, or climbed down in a slow and uncoordinated fashion (Figure 4D). NestinCre;Rai1CKO mice also showed decreased latency to fall in a wire hang test (Figure 4E). Interestingly, Rai1<sup>−/−</sup> mice showed a decreased latency to fall, suggesting that performance in the wire hang test is more sensitive to Rai1 dosage than the pole test.

Next, we examined whether the mice had deficits in tests purported to assess anxiety, sociability, and cognition. We measured anxiety-like behavior using the elevated plus maze and found that the time NestinCre;Rai1CKO mice spent in open and closed arms was not statistically different from their control littermates (Figures S4J and S4K). Consistently NestinCre;Rai1CKO and control mice exhibited no difference in exploring the periphery versus center in open field test (Figure S4G). NestinCre;Rai1CKO mice also appeared normal in sociability and social discrimination assays (Figures S4L and S4M). However, NestinCre;Rai1CKO mice exhibited a significantly reduced tendency to investigate the new arm in the Y-maze test (Figure S4N), suggesting a spatial working memory deficit.

Finally, in a Pavlovian fear-conditioning task that tests learning and memory, control mice progressively developed a freezing response to a tone followed by a shock, whereas NestinCre;Rai1CKO mice had low freezing behavior after repeated tone-shock pairings during training (Figure 4F). As a result, these mice had reduced total freezing on the training day compared to littermates of other genotypes (Figure 4G). It is unlikely that these defects were caused by deficits in audition or pain sensation, as NestinCre;Rai1CKO mice exhibited normal pain responses in the hot plate assay and normal auditory startle responses.
Figure 4. Pan-neural Loss of Rai1 Causes SMS-like Phenotypes in Mice
(A) Survival curves for male and female control (Rai1+/+, NestinCre+;Rai1+/+, or NestinCre;Rai1−/−; n = 26, black line), whole-body heterozygous knockout (Rai1+/− or Rai1/flox); n = 27, gray line, overlaying the controls), whole-body heterozygous knockout plus NestinCre (NestinCre;Rai1+/−; n = 5, blue line), heterozygous NestinCre knockout (NestinCre;Rai1+/−; n = 12, pink line, overlapped with controls), and homozygous NestinCre knockout (NestinCre;Rai1CKO; n = 12, red line) mice. Most NestinCre;Rai1CKO mice die in early-to-mid adulthood, significantly younger than mice in all other groups (Log-rank test, p < 0.0001).

(B) Mean (±SEM) body weights over time of male (left) and female (right) control (n = 12 males and 16 females), NestinCre;Rai1+/− (n = 5 males and 0 females), NestinCre;Rai1+/− (5 males and 7 females), and NestinCre;Rai1CKO (n = 7 males and 5 females) mice. Female mice lacking one or both copies of Rai1 in the Nestin+ lineage or one copy in all tissues become obese (two-way ANOVA: genotype and genotype × time interaction, p < 0.001 for females and p > 0.05 for males).

(C) Rai1 disruptions do not affect the distance traveled in an activity chamber. Each dot represents a single mouse. Data are mean ± SEM.

(D) Time (mean of three trials per mouse) required by each mouse to climb down a wooden pole after being placed at the top. Mice that fell or slipped off the pole were given a score of 60 seconds (a). NestinCre;Rai1CKO mice slipped or fell down the pole, or climbed down very slowly, whereas heterozygous knockout and control mice agilely descended the pole in less than 6 s.

(E) Mean (±SEM) time mice were able to hang inverted from a wire grid before dropping; trials were terminated after 150 s (a), in which case mice were given scores of 150 s. Rai1 heterozygous mice and NestinCre;Rai1CKO mice show deficits in this task.

(F) Mean (±SEM) percentage time spent freezing during training on the day the tone was presented by itself in a different context on the subsequent day (cued recall). NestinCre; Rai1CKO mice exhibit a drastic deficit in developing a freezing response compared to littermates of other genotypes.

(G) Mean (±SEM) percentage time spent freezing on all 3 days (training, cued recall, and contextual recall) of a trace fear-conditioning task. Values reflect total freezing with baseline period removed from the average. Statistics: n.s., not significantly different, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, Tukey’s post hoc test following a significant ANOVA. See also Figure S4.
day but still reduced compared to controls (Figure 4G). They also showed reduced freezing in the conditioning context without the tone (context recall) (Figure 4G). Therefore, despite modest steady-state transcriptomic changes, NestinCre;Rai1CKO mice showed phenotypes that mimic many SMS symptoms, including deficits in body weight homeostasis, motor skills, and associative learning and memory.

**Rai1 Loss in Subcortical Excitatory Neurons Causes Motor Dysfunction**

As Rai1 is widely expressed in the brain (Figure 1E), it is possible that (1) each phenotype is caused by loss of Rai1 in a distinct group of cells (Figure 5A); (2) each cell type partially contributes to many phenotypes (Figure 5B); (3) most phenotypes are caused by loss of Rai1 in one critical group of cells (Figure 5C); or a combination of the above. To distinguish between these possibilities, we selected three SMS-like phenotypes (motor, learning, and obesity) exhibited by NestinCre;Rai1CKO mice and conducted a cell-type screen to determine whether loss of Rai1 from specific cell populations would recapitulate these deficits. Specifically, one or both copies of Rai1 was removed using (1) Gad2Cre that targets most GABAergic inhibitory neurons (Taniguchi et al., 2011), (2) Emx1Cre that targets cortical and hippocampal excitatory neurons and glia (Gorski et al., 2002), (3) mGfapCre that targets astrocytes and subsets of adult neural progenitors (Garcia et al., 2004), and (4) Vglut2Cre that targets subcortical excitatory neurons (Vong et al., 2011). Immunostaining confirmed that Rai1 was selectively deleted in Cre-expressing cells (Figures S5A–S5E; see Figure S6 for phenotypes other than motor, learning, and obesity analyzed in these chronic knockouts).

We first tested which cell types were responsible for the motor phenotypes seen in NestinCre;Rai1CKO mice. All conditional mutants were similar in weight to their control littersates at the time of behavioral assays (Figure SSF). In the pole test, the time required to descend was increased only in Vglut2Cre;Rai1CKO mice (Figures S5D–S5G). In the wire hang test, Gad2Cre;Rai1CKO mice performed slightly better than controls (Figure S5H), while removing one or both copies of Rai1 from Emx1+ or mGfap+ cells did not interfere with performance (Figures 5I and 5J). By contrast, Vglut2Cre;Rai1CKO mice developed poor motor functions, as exemplified by a decreased latency to fall in the wire hang test (Figure 5K). Removing one copy of Rai1 from the Vglut2+ neurons caused an intermediate phenotype in the wire hang test (Figure 5K). Thus, Rai1 function in Vglut2+ excitatory neurons is essential for proper motor function and is dosage sensitive in a subset of the assays.

**Rai1 Loss in Either GABAergic Neurons or Subcortical Excitatory Neurons Disrupts Fear Conditioning**

Fear conditioning is regulated by distributed networks in the brain involving excitatory and inhibitory neurons (Letzkus et al., 2015). We found that Emx1Cre;Rai1CKO and mGfapCre;Rai1CKO mice did not show learning deficits (Figures 5M and 5N). However, both Gad2Cre;Rai1CKO and Vglut2Cre;Rai1CKO mice exhibited reduced freezing during training (Figures 5L and 5O). All conditional mutants and controls responded to shocks during training with increased locomotion or jumping, similar to control mice (data not shown). Both Gad2Cre;Rai1CKO and Vglut2Cre;Rai1CKO mice exhibited normal pain sensitivity in a hot plate assay, suggesting that the fear conditioning phenotypes were due to learning deficits rather than secondary consequences of defective pain sensitivity. Additionally, Vglut2Cre;Rai1CKO and Gad2Cre;Rai1CKO mice both showed reduced cued- and contextual-memory recall (Figures S5L and 5O), mimicking the NestinCre;Rai1CKO mice. Together, these results indicate that Rai1 is required in both Gad2+ inhibitory and Vglut2+ subcortical excitatory neurons for associative learning and memory.

**Rai1 Loss in Subcortical Excitatory Neurons, Sim1+ Cells, or SF1+ Cells Causes Obesity**

A prominent feature of SMS is truncal obesity, which develops in the young adolescent stage (Burns et al., 2010). Our analysis of NestinCre;Rai1CKO mice suggested that Rai1 is deleted from both nuclei (Figures S7A–S7D). Unlike NestinCre;Rai1CKO mice, Vglut2Cre;Rai1CKO mice showed increased body weight when compared to control littersates in both females (Figures 6A–6D) and males (Figures S7A–S7D). Unlike NestinCre;Rai1CKO mice, Vglut2Cre;Rai1CKO mice did not show premature lethality, and thus weight analysis did not suffer from the complication of weight loss prior to death, as in NestinCre;Rai1CKO mice. Male and female Vglut2Cre;Rai1CKO mice became obese at 9 and 7 weeks of age, respectively (Figures 6D and S7D) and were 56% (males) and 116% (females) heavier than control littersates at 20 weeks of age. Mice losing one copy of Rai1 in the Vglut2+ neurons were moderately overweight (males: 16% overweight, female: 29% overweight). By contrast, the body weight of Emx1Cre;Rai1CKO, Gad2Cre;Rai1CKO, and mGfapCre;Rai1CKO mice was not significantly different from their control littersates, highlighting that Rai1 is specifically required for Vglut2+ subcortical excitatory neurons to regulate energy homeostasis.

Next, we asked which subtypes of Vglut2+ neurons regulate body weight. The hypothalamus is an important brain center for controlling appetite and energy expenditure (Saper and Lowell, 2014). Within the hypothalamus, Vglut2Cre is expressed in the posterior hypothalamus, dorsal nucleus of hypothalamus, lateral hypothalamus, paraventricular nucleus of hypothalamus (PVH), ventromedial nucleus of hypothalamus (VMH), and a subset of POMC neurons in the arcuate nucleus (Vong et al., 2011). The VMH and PVH have emerged as critical hypothalamic nuclei that control feeding (Dhillon et al., 2006; Tong et al., 2007). We generated female Emx1Cre;Rai1CKO mice in which Sim1Cre was preferentially deleted in the PVH or VMH, respectively, whereas in Vglut2Cre;Rai1CKO mice Rai1 was deleted from both nuclei (Figures S7E and S7F). Notably, Sim1Cre;Rai1CKO mice were 57% heavier than their control littersates at 20 weeks of age.
Figure 5. Rai1 Is Required in Specific Neuronal Types for Motor Functions and Learning
(A–C) Three models for the relationship between Rai1’s function in specific cell types and its organismal functions. See text for details. (D–K) Latency to descend in the pole test (D–G) and fall in the wire hang test (H–K) for mice in which Rai1 was deleted in specific cell types as indicated. Data are mean ± SEM.
(L–O) Removal of Rai1 in either Gad2+ (L) or Vglut2+ (O) neurons, but not in Emx1+ (M) or mGfap+ (N) cells, impairs performance in a fear-conditioning task. Data are mean ± SEM.
Statistics: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, Tukey’s post hoc test following a significant ANOVA. See also Figures S5 and S6.
SF1Cre;Rai1CKO mice were 27% heavier than controls at the same age (Figure 6F). Removing one copy of Rai1 from the Sim1+ cells generated an intermediate overweight phenotype (12.7% heavier than controls at 20 weeks). Mice losing one copy of Rai1 from the SF1+ cells were equally obese as SF1Cre;Rai1CKO mice, suggesting that Sim1+ and SF1+ cells are differentially sensitive to Rai1 dosage. Therefore, both Sim1+ and SF1+ cells contribute to the obesity phenotype observed in Vglut2Cre;Rai1CKO mice, likely due to loss of Rai1 in PVH and VMH neurons, respectively, although we cannot rule out the contribution of Sim1+ or SF1+ cells outside the PVH or VMH.

Given that Sim1+ cells played a more dominant role than SF1+ cells in obesity due to Rai1 loss, we next aimed to identify the source of increased body weight in Sim1Cre;Rai1CKO mice. We quantified body composition with dual-energy X-ray absorptiometry (DEXA) and found that Sim1Cre;Rai1CKO mice showed significantly increased lean and fat mass (Figures 6G and 6H). Consistent with DEXA results, the weight of each dissected fat pad, including brown adipose tissue (BAT), was greater in Sim1Cre;Rai1CKO mice (Figure 6I). These data indicate that Sim1Cre;Rai1CKO mice developed increased adiposity. In addition to obesity, SMS patients show higher levels of total cholesterol, HDL, LDL, and triglycerides (Smith et al., 2002). Consistent with

(Figure 6E), whereas SF1Cre;Rai1CKO mice were 27% heavier than controls at the same age (Figure 6F). Removing one copy of Rai1 from the Sim1+ cells generated an intermediate overweight phenotype (12.7% heavier than controls at 20 weeks). Mice losing one copy of Rai1 from the SF1+ cells were equally obese as SF1Cre;Rai1CKO mice, suggesting that Sim1+ and SF1+ cells are differentially sensitive to Rai1 dosage. Therefore, both Sim1+ and SF1+ cells contribute to the obesity phenotype observed in Vglut2Cre;Rai1CKO mice, likely due to loss of Rai1 in PVH and VMH neurons, respectively, although we cannot rule out the contribution of Sim1+ or SF1+ cells outside the PVH or VMH.
Figure 7. Mechanisms Underlying Hypothalamic Dysfunction upon Rai1 Deletion

(A) Daily food intake of normal chow-fed mice (averaged over 7 days, mean ± SEM). Sim1Cre;Rai1CKO mice show significantly increased daily food intake (n = 8 for each genotype).

(B) Beam-break counts (mean ± SEM) indicating a decrease of horizontal locomotor activity in 30-week-old Sim1Cre;Rai1CKO mice (n = 8 for each genotype).

(C) Respiratory exchange rate (mean ± SEM) as calculated by VCO2/VO2 is not significantly different among groups (n = 8 for each genotype).

(D) Expression of Rai1 exon 3 and Bdnf are downregulated in the Vglut2Cre;Rai1CKO hypothalamus, as revealed by qRT-PCR. Data are mean ± SEM (n = 3).

(E) Volcano plot of RNA-seq results of control and Vglut2Cre;Rai1CKO hypothalamus. Red dots indicate genes that were further validated.

(F) qRT-PCR showing that the mRNA expression of Pcdh20, Htr2c, and Sema3a are commonly downregulated in the NestinCre;Rai1CKO cortex, Gad2Cre;Rai1CKO striatum (both 3 weeks and 12 weeks), and Vglut2Cre;Rai1CKO hypothalamus. Data are mean ± SEM (n = 3).

(G) UCSC browser tracks of mouse Bdnf (left) and Htr2c (right) genomic loci annotated with Rai1 ChIP-seq and input signals (orange, our data from cortex), histone marks (gray, ENCODE cortex data), and our RNA-seq signals from the control (light blue) and Vglut2Cre;Rai1CKO (dark blue) hypothalamus. Red arrows
that and confirmed by qRT-PCR (Figure 7D) a previous observation See also Figure S7.

Statistics: n.s., not significant, p > 0.05, *p < 0.05, and **p < 0.01, unpaired t test. 
depressed Bdnf may underlie the severe obesity in significantly affected. Together, these data suggest that hyperphagia decreased energy expenditure, or their combination. To distinguish among these possibilities in Sim1Cre;Rai1CKO mutants became obese. and control littermates at 8 weeks of age, before the conditional knockout, and Vglut2+ neurons—which include both Sim1+ and SF1+ cells—exhibit an intermediate heterozygous phenotype that is as severe as homozygous loss of Rai1 in either Vglut2+ or Gad2+ neurons, and obesity likely results from a combination of Rai1 deficiency in hypothalamic PVH and VMH neurons. Indeed, loss of Rai1 in Sim1+ and SF1+ cells alone results in less severe weight gain than if Rai1 is removed from all Vglut2+ neurons, suggesting an involvement of additional cell types. Rai1 loss in Vglut2+ neurons causes

Potential Mechanisms Underlying Obesity in Rai1 Mutants

Obesity can be caused by increased food intake, decreased energy expenditure, or their combination. To distinguish among these possibilities in Sim1Cre;Rai1CKO mice, we monitored their home cage food intake for a week. We found that they displayed hyperphagia, with a 47% increase in average daily food intake compared to control littermates (Figure 7A), and decreased horizontal locomotor activity over a 48 hr period (Figure 7B). Energy expenditure after normalization to lean body mass (Figures 7H–7J) and respiratory exchange rate (Figure 7C) was not significantly affected. Together, these data suggest that hyperphagia may underlie the severe obesity in Sim1Cre;Rai1CKO mice.

To identify the misregulated genes that may explain Rai1-associated obesity and increased food intake, we performed RNA-seq using hypothalamus isolated from Vglut2Cre;Rai1CKO and control littermates at 8 weeks of age, before the conditional mutants became obese. Rai1 mRNA was downregulated by ~50% in the Vglut2Cre;Rai1CKO hypothalamus (Figure 7D), consistent with the fact that Vglut2+ excitatory neurons only account for a subset of hypothalamic cells. Due to this dilution effect, and potential gene expression heterogeneity in Vglut2+ neurons in different hypothalamic nuclei, the magnitude and number of differentially expressed genes in Vglut2+ neurons are likely to be an underestimation. Still, we detected by RNA-seq (Figure 7E) and confirmed by qRT-PCR (Figure 7D) a previous observation that Bdnf is downregulated in Rai1 heterozygous mice (Burns et al., 2010). In addition, we identified several genes involved in cell-cell communication, such as Htr2c (encoding the serotonin receptor 2c), Pcdh20 (encoding a cell adhesion molecule protocadherin), and Sema3a (encoding an axon guidance protein), that were downregulated not only in Vglut2Cre;Rai1CKO hypothalamus (Figure 7E), but also in the Rai1 mutant cortex and striatum (Figure S3G and Table S5). We confirmed these downregulations independently by qRT-PCR (Figure 7F). Interestingly, both Bdnf and Htr2c mutant mice exhibit over-feeding behaviors that lead to obesity (Kemere et al., 2000; Tecott et al., 1995), and Rai1 binds to the promoters of both Bdnf and Htr2c (Figure 7G). Together, our hypothalamus RNA-seq data suggest that decreased Bdnf and Htr2c expression due to Rai1 loss may contribute to obesity.

DISCUSSION

Designing treatment strategies for neurodevelopmental disorders associated with genetic mutations requires a comprehensive understanding of their genetic causes, molecular functions of the affected genes, and the cell types that underlie different symptoms (Kaiser and Feng, 2015; Mullins et al., 2016; Wells et al., 2016; Zoghbi and Bear, 2012). In this study, we present an extensive functional analysis of Rai1—a causal gene for two syndromic neurodevelopmental disorders (SMS and PTLS)—at molecular and behavioral levels. Our data show that Rai1 is broadly expressed in postmitotic neurons and binds to promoter regions to positively regulate the expression of target genes, many of which function in circuit assembly and neuronal communication. Each SMS phenotype may be caused by loss of Rai1 in multiple non-overlapping cell types (Figure 8). Our finding that Vglut2+ subcortical excitatory neurons are major contributors of most phenotypes further highlights the importance of targeting these neurons for therapeutic intervention.

Our systematic conditional knockout analyses provide new insights into the neural functions of Rai1. First, different cell types are differentially sensitive to loss of Rai1. For example, we did not detect any phenotypes in fear conditioning (Figure 5) or social interactions (Figure S6) resulting from removal of Rai1 from the Emx1+ cells, which includes all excitatory neurons in the cortex and hippocampus, despite the prominent Rai1 expression in those cells. This is not because Emx1+ cells are not required in these behavioral tasks. For instance, it is well established that hippocampal function is required in contextual fear conditioning (Toyote et al., 2015), and social interactions engage frontal cortex (Barak and Feng, 2016). These data suggest that Rai1 does not have a general, house-keeping function that is required for every cell type. Rather, Rai1 has more specific roles in certain cell types.

Second, dosage sensitivity of Rai1 differs for cell types and phenotypic assays. For example, for obesity, Sim1+ cells exhibit at most a mild heterozygous phenotype, SF1+ cells exhibit a heterozygous phenotype that is as severe as homozygous knockout, and Vglut2+ neurons—which include both Sim1+ and SF1+ cells—exhibit an intermediate heterozygous phenotype. Within Vglut2+ neurons, losing one copy of Rai1 causes a motor defect in the wire hang test but not pole test. Further, Vglut2+ and Gad2+ neurons necessary for learning are only dysfunctional when both copies of Rai1 were deleted. The lack of some phenotypes after losing one copy of Rai1, which more closely mimics the human syndrome, may be due to differences in physiology of mice and humans or due to insufficient sensitivity of assays in mice to mimic human conditions.

Third, Rai1 function in multiple cell types contributes to SMS-like phenotypes in mice (Figure 8). Learning deficits originate from loss of Rai1 in either Vglut2+ or Gad2+ neurons, and obesity likely results from a combination of Rai1 deficiency in hypothalamic PVH and VMH neurons. Indeed, loss of Rai1 in Sim1+ and SF1+ cells alone results in less severe weight gain than if Rai1 is removed from all Vglut2+ neurons, suggesting an involvement of additional cell types. Rai1 loss in Vglut2+ neurons causes
Figure 8. Cell-Type Specificity of SMS-like Phenotype in Mice
Schematic summary of the neurobehavioral phenotypes due to loss of Rai1, and the corresponding cell types that require Rai1 identified in this study. Listed are four identified phenotypes observed in NestinCre;Rai1CKO mice from our analyses. Whereas loss of Rai1 in Emx1+ and Gfap+ cells does not lead to detectable phenotypes, Rai1 function in these cell types can contribute to untested phenotypes or have additive/synergistic effects when combined with other cell types. Rai1 loss in Vglut2+ cells is a major contributor to learning, obesity, and motor phenotypes, and Rai1 loss in Gad2+ cells leads to learning deficits. Rai1 in Sim1+ cells, which include Vglut2+ PVH neurons, is a dominant contributor to over-feeding and obesity phenotypes. Rai1 in SF1+ cells, including Vglut2+ VMH neurons, plays a less prominent role in regulating body weight.

Given the strong phenotypes of Rai1 mutant mice and the large number of Rai1 target genes, our ChIP-seq analysis, loss of Rai1 caused a surprisingly modest change in the transcriptome both in the number of genes and the magnitude of mRNA levels. One possible explanation is that besides moderately promoting gene expression, Rai1 may regulate transcription in response to specific stimuli or changes in neuronal activity, which is not easily captured by sampling whole-tissue steady-state level of mRNAs. For example, several chromatin modifications have more important roles during dynamic gene activation and repression than steady-state expression (Weiner et al., 2012). Indeed, Rai1’s role in context-dependent transcription will be an interesting topic of future research. Another possible explanation is that misregulation of a small number of key Rai1 target genes is sufficient to account for SMS symptoms. For example, we detected downregulation of Bdnf and Htr2c expression in Vglut2Cre, Rai1CKO hypothalamus. Given the important roles for hypothalamic Bdnf (An et al., 2015; Xu and Xie, 2016) and Htr2c (Nonogaki et al., 1998; Tecott et al., 1995) in regulating energy homeostasis, the severe obesity observed in Rai1 mutants may result from downregulation of one or both signaling pathways. In this regard, our study suggests an avenue for therapeutic intervention, at least for obesity, by restoring these signaling pathways. Given that there is an FDA-approved Htr2c agonist for treating obesity (Colman et al., 2012), this strategy can be tested experimentally.

EXPERIMENTAL PROCEDURES

Mouse Behavioral Assays
Male mice were housed in groups on an inverted 12/12 hr light/dark cycle with ad libitum access to food and water and were tested between 6 and 10 weeks of age. Behavioral testing was conducted during the mouse’s subjective night, except the hot plate assay, which was performed during the subjective day. Mice were habituated to handling for 3 days prior to the onset of the first behavioral tests. Behavioral tests were conducted in multiple cohorts of mice; each cohort followed the same sequence of behavioral tests as listed in Supplemental Experimental Procedures. Experimenters were blind to mouse genotype during testing.

Detailed description of mouse behavioral assays, as well as additional methods are described in Supplemental Experimental Procedures, including mouse husbandry and handling, generation of the Rai1-Tag mice and Rai1-flox mice, mouse genotyping, protein expression and purification, in vitro binding assay, generation of Rai1 antibody, in situ hybridization followed by immunostaining, immunostaining and antibodies, chromatin immunoprecipitation-sequencing (ChIP-seq) and ChiP-qPCR, RNA-seq and qRT-PCR, data analysis for ChiP-seq and RNA-seq, energy homeostasis analyses, and reagents and data sharing.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and nine tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.09.019.

AUTHOR CONTRIBUTIONS

helped design and provided reagents for the in vitro chromatin assay. W.-H.H. and L.L. wrote the paper, with contributions from all authors.

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REFERENCES


