Correct developmental expression level of Rai1 in forebrain neurons is required for control of body weight, activity levels and learning and memory

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Received August 21, 2013; Revised and Accepted November 6, 2013

Potocki–Lupski syndrome (PTLS) is a genomic disorder associated with an ∼3 Mb duplication in 17p11.2. Clinical features include leanness, intellectual disability, autistic features and developmental deficits. RAI1 gene dosage is associated with the PTLS phenotypes. To understand where and when Rai1 overexpression is detrimental, we generated a mouse that over-expresses Rai1 conditionally in forebrain neurons (I-Rai1). Phenotypic characterization of I-Rai1 mice showed significant underweight, hyperactivity and impaired learning and memory ability compared with wild-type littermates. Doxycycline administration can turn off the transgene expression allowing the restoration of Rai1 normal expression levels. When the transgene was turned off from conception to 3 months of age, no phenotypic differences were observed between I-Rai1 and their wild-type littermates. Surprisingly, we found that turning off the transgene expression before the onset of the phenotypes (1–3 months) or after the onset of the phenotypes (3–5 months) cannot prevent nor reverse the phenotypic outcomes. Our results indicate that Rai1 dosage in forebrain neurons is critical during the development and is related to body weight regulation, activity levels and learning and memory.

INTRODUCTION

Potocki–Lupski syndrome (PTLS) (OMIM# 610883) is caused by an ∼3 Mb duplication of the 17p11.2 region and is associated with neurodevelopmental and behavioral phenotypes. Clinical presentations of PTLS patients include mild to severe intellectual disability, developmental delay, autism, hyperactivity, infantile hypotonia, apnea and structural cardiovascular anomalies (1,2). The incidence of this genomic disorder is estimated to be ∼1 in 25 000. The majority of PTLS cases reported to date involve de novo duplications (3); however, a transmission from a mosaic mother to her son was also reported (4).

Around 23 genes are duplicated in the vast majority of PTLS patients. However, by analyzing the smallest region of overlap in patients carrying different size duplications, a critical genomic interval of 1.3 Mb that contains 15 known genes (2) was defined. In 2010, Zhang et al. (5) analyzed 74 PTLS duplications and further defined a 125 kb interval which only contains the RAI1 gene, strongly suggesting that RAI1 was indeed the major dosage sensitive gene causing PTLS in human patients.

Additional evidence that Rai1 is the main responsible gene for the PTLS clinical presentation came from mouse studies. Human chromosome 17p11.2 is syntenic to the 32–34 cM region of murine chromosome 11. A PTLS mouse model (heterozygous

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for a duplication in mouse chromosome 11 syntenic region) was generated using chromosomal engineering (Dp(11)17/+) (6). Dp(11)17/+ mice recapitulated several clinical manifestations of PTLS syndrome, such as mild motor abnormalities, under-weight, hyperactivity, anxiety-like behavior and learning and memory deficits (6–9). By analyzing compound heterozygous Dp(11)17/Rai1−/− mice—where only Rai1 gene dosage is restored, but the other 22 genes on the interval remained triplicated—most PTLS phenotypes observed in Dp(11)17/+ were rescued, strongly supporting a crucial role of Rai1 in the PTLS clinical presentation (8). Rai1 is predominantly expressed in neurons of the hippocampus, striatum, cerebral cortex and in the Purkinje cell layer of the cerebellum (10).

RAI1/Rai1 is a nuclear protein with transcription factor activity (11–13). We have demonstrated by in vitro functional studies that the Rai1 protein has two main domains, the N-terminal half that is related to the transcription factor activity and the C-terminal half that can direct the protein into the nucleus (12). Rai1 protein was found to be enriched in chromatin bound fraction in human lymphoblastoid cells (13) and it was also reported that Rai1 is strongly enriched on chromatin in interphase HeLa cells (14).

Despite all the data relating Rai1 gene dosage with the PTLS phenotypes both in human and mouse models, it is not yet clear whether the requirement for a correct expression of Rai1 is permanent and pan-neuronal and if there is a window of time-specific brain region that is especially susceptible to altered Rai1 expression. In this study, we tested the consequences of overexpressing Rai1 in specific brain regions, and determined whether it is possible to prevent or reverse the neurological phenotypes by restoring Rai1 normal expression level during different developmental stages.

RESULTS

Generation of transgenic mice with an inducible wild-type Rai1 allele

As a first step toward the generation of transgenic mice that overexpress wild-type Rai1 in forebrain neurons, we constructed a Rai1-pBi-eGFP (or pBi-Rai1) vector that contains a bidirectional promoter designed to allow the simultaneous expression of both Rai1 (wild-type) and eGFP controlled by one central tetracycline-responsive element (Bi-tetO promoter) (Supplementary Material, Fig. S2A). In order to differentiate the transgenic protein from the endogenous one, an HA tag was added to the C-terminal coding region of transgenic Rai1. The HA tag was previously shown not to interfere with Rai1 subcellular localization or function by study by in vitro studies (12). Rai1 transcript will not be expressed unless tetracycline-inhabitable transactivator (tTA) is present and bound to the Bi-tetO promoter. The correct functioning of the vector was tested in vitro in Neuro-2a cells prior to microinjections. As predicted, the expression of the Rai1-HA and eGFP proteins was only observed when co-transfection with a vector expressing tTA was performed (Supplementary Material, Fig. S1). Further, Rai1-HA and eGFP expression was abolished when the transfected cells were treated with Dox, a tetracycline derivative that binds to and inactivate tTA transactivation activity (Supplementary Material, Fig. S2). The presence of Rai1-HA protein was checked both by immunofluorescence (Supplementary Material, Fig. S1A) and western blot analysis (Supplementary Material, Fig. S1C).

Next pBi-Rai1 microinjections were done in pronuclei of C57B6/6J X CBA/J zygotes and six transgenic founders were obtained. Southern blot analysis showed that the transgene copy number ranged from 2 to 26, and that a single place of insertion for the transgene was present in all the analyzed founders (Supplementary Material, Fig. S3). By crossing the pBi-Rai1 mice with mice expressing tTA under the control of the forebrain-specific calcium-calmodulin-dependent kinase 2a (Camk2a) promoter (Camk2a-tTA mice) (15), we obtained double transgenic-Rai1 (I-Rai1) mice (Supplementary Material, Fig. S2A) that overexpress Rai1 specifically in mouse forebrain neurons. Two double transgenic lines were analyzed in this study: I-Rai1463 and I-Rai1479 carrying six and two copies of the transgene, respectively (Supplementary Material, Fig. S3).

Specific expression of transgenic Rai1

As shown in the in vitro studies, we expected that in I-Rai1 mice, both eGFP and Rai1-HA expression will be concerted. To test this, primary cultured neurons derived from I-Rai1463 and I-Rai1479 mice brains were obtained. eGFP was directly observed by its own fluorescence, while the presence of transgenic Rai1 was assessed with anti-HA immunofluorescence. As can be seen in Supplementary Material, Figure S1B and C, most of the cells are expressing both transgenes (94.5% for I-Rai1463 and 92% for I-Rai1479), indicating that the eGFP fluorescence can be utilized as a marker of tissue-specific expression of Rai1-HA. In addition, by immunofluorescence against HA, we saw that 100% of the counted cells presented the correct nuclear subcellular localization of the Rai1 transgenic protein in both I-Rai1 lines (200 cells were counted for each line) (Supplementary Material, Fig. S1B).

In order to determine the in vivo expression levels of the Rai1 transgene, cDNA was generated from RNA extracted from different brain regions of I-Rai1463, I-Rai1479 and wild-type littermates. qPCR analysis was performed with primers that can detect both the transgenic and the endogenous Rai1 mRNA. The expression levels of the wild-type were set up as 1 for normalization. As can be seen in Figure 1A, the I-Rai1463 had an ~4-fold overexpression of Rai1 in olfactory bulb compared with ~1.5 fold overexpression for line I-Rai1479, while both lines presented similar levels of overexpression, ~3-fold, in cortex and hippocampus. To assess the transgenic protein expression, the hippocampus of wild-type and I-Rai1463 were dissected and the protein extracts were obtained and separated in a 7.5% SDS–PAGE gel followed by western blot analysis with anti-HA antibody. A band at the expected molecular weight (~250 kDa) was observed for the double transgenic animals and it was absent in the wild-type littermates confirming the presence of the transgenic protein in the I-Rai1463 brain (Fig. 1B). It was reported that Rai1 is mainly bound to the chromatin in human lymphoblastoid cells (13). To test if the transgenic protein was also mainly chromatin bound, we performed cell fractionation experiments using I-Rai1463 hippocampus to see the localization of Rai1-HA in vivo. We found that Rai1-HA is enriched in the chromatin fraction (Fig. 1C), suggesting that
the transgenic protein has a normal intranuclear localization in vivo.

To determine the specific-brain areas where the transgene is expressed, we used eGFP fluorescence as a marker (Fig. 2). Sagittal and coronal brain slides of both I-Rai1 lines were obtained and eGFP fluorescence was analyzed by confocal microscopy. As can be seen in Figure 2A, the expression of the transgene is forebrain specific. eGFP expression is observed in hippocampus, cerebral cortex, striatum, amygdala and olfactory bulb (Fig. 2B). In contrast, no eGFP expression was observed in cerebellum and brain stem (Fig. 2A) or in any region of the wild-type or pBi-Rai1 littermates (Supplementary Material, Fig. S4). Immunofluorescence using anti-NeuN, to label neurons exclusively, was performed and transgene expression was found restricted to the neurons, as expected for the Camk2a promoter (Fig. 2C). Anti-GFAP was used to label glial cells and no eGFP expression was observed in GFAP-positive cells (Fig. 2D). In order to understand the phenotypic consequences of overexpressing Rail in forebrain neurons independent of the transgene integration site, we phenotypically characterized these two independent I-Rai1 lines: I-Rai1463 and I-Rai1479.

I-Rai1 mice have normal viability and neurological reflexes, but lower vocalization and reduced body weight

To address if the transgenes impaired viability, we analyzed the number of mice obtained from the four different genotypes resulting from a pBi-Rai1/+ X Camk2a-tTA/+ mating (wild-type, pBi-Rai1/+, Camk2a-tTA/+ , I-Rai1). The expected Mendelian ratio was observed for both I-Rai1 lines. For the I-Rai1463 line from a total of 125 pups born, 29 were I-Rai1463, 26 were pBi-Rai1463/+ , 32 were Camk2a-tTA/+ and 38 had a wild-type genotype, without significant difference compared with the expected Mendelian rate ($P = 0.47$), for 479 line, from a total of 123 pups, 30 were I-Rai1479, 34 were Camk2a mice, 25 were wild-type and 34 were pBi-Rai1479/+ mice, also following the Mendelian rate ($P = 0.61$).

General characteristics were examined and no significant difference was observed in coat condition, presence of barbering, piloerection and presence of whiskers between I-Rai1 mice and the controls littermates. Visual placing reflex and reactions to a gentle cotton swab touch to the whiskers were also normal. Interestingly enough, a significantly lower percentage of vocalization was observed in I-Rai1 mice, 17 and 8% in I-Rai1463 and I-Rai1479 respectively, compared with 29% in wild-type mice ($P < 0.01$ for both lines) (Table 1). No overt seizures were observed in the I-Rai1 mice. In addition, Camk2a-tTa/+ or pBi-Rai1/+ mice did not present any of these phenotypes.

We analyzed the weight curve of our two independent I-Rai1 mouse lines finding that both of them were underweight when compared with the wild-type littermates. I-Rai1463 mice started to show significant underweight at 4 months of age, $30.3 \pm 1.4$ g when compared with the wild-type littermates $34.3 \pm 1.2$ g ($P = 0.05$) (Fig. 3A). The same was true for the I-Rai1479 that started to show significant underweight at 2 months of age, $22.9 \pm 1.0$ g, compared with the wild-type littersates $26.4 \pm 0.77$ g ($P = 0.02$) (Fig. 3A). No significant difference in weight was observed between the two I-Rai1 mouse lines. Single transgenic mice, pBi-Rai1463, pBi-Rai1479 and Camk2a-tTa, showed no significant weight difference when compared with wild-type littermates (Supplementary Material, Fig. S5A). At 6 months of age, the mice were sacrificed and the different organs weights were recorded. As can be seen in Figure 3B, the abdominal fat weight for both I-Rai1 lines was significantly less than in wild-type animals, $0.58 \pm 0.22$ g for 463 ($P = 0.03$) and $0.44 \pm 0.14$ g for 479 ($P = 0.002$) compared with $1.2 \pm 0.1$ g in wild-type mice. Cerebellum, spleen, heart and kidney were also examined and no significant weight difference was observed (Supplementary Material, Fig. S4).
I-Rai1 mice are hyperactive but anxiety behavior was not observed

To determine whether the overexpression of Rai1 in forebrain neurons was sufficient to cause the hyperactivity and increased anxiety observed in the PTLS mouse model, we study the I-Rai1 mice in the open field and plus maze tests. In the open field, a significant difference was observed in the total distance travelled between wild-type (3067 ± 300 cm) versus I-Rai1463 mice (5312 ± 858 cm) (P = 0.025) and I-Rai1479 mice (6879 ± 2116 cm) (P = 0.01) (Fig. 3C). No difference was found between the two I-Rai1 lines (P = 0.42) or between wild-types versus single transgenic mice (Supplementary Material, Fig. S5). These results indicate that I-Rai1 mice are hyperactive.

The central/total distance ratio, an indicator of anxiety-like behavior, was found to be similar between I-Rai1 and wild-type controls, for both I-Rai1 lines, suggesting normal levels of anxiety in this paradigm (Fig. 3D). Subsequently, the plus maze test was performed to these mice. The plus maze is an independent test to assess anxiety-like behaviors. As shown in Figure 3E, wild-type mice spent significantly more time in the close arms (47 ± 6% of the time) than in the open arms (23 ± 4% of the time) (P = 0.005). I-Rai1463 and I-Rai1479 mice also preferred to stay in the close arms (I-Rai1463 55 ± 3 versus 22 ± 3%, P < 0.01, and I-Rai1479 50 ± 4 versus 25 ± 4%), showing there is no significant difference between genotypes in the time spent in the open or the close arms, clearly indicating that...
Learning and memory ability are impaired in I-Rai1 mice

We then evaluated learning and memory ability in these mice utilizing the Pavlovian paradigm with the fear conditioned test to contextual and sound cued fear. Contextual-related memory is hippocampus dependent, whereas sound cued fear memory is hippocampus independent (16). Briefly, animals were trained by being placed inside a closed chamber and exposed to a 30 s tone and a paired foot shock. Their short-term contextual memory was tested 2 h after training by placing them back to the same chamber and their sound cued fear memory was tested after another hour by placing them to a modified chamber with different context and odor. Interestingly, I-Rai1463 and I-Rai1479 mice showed significant less freezing both in contextual (I-Rai1463 24.49%, I-Rai1479 21.88% versus wild-type 42.26%, $P < 0.05$) for both transgenic lines and sound cue tests (I-Rai1463 8.24%, I-Rai1479 13.94%, versus wild-type 23.76%, $P < 0.05$) (Fig. 3F and G), indicating that their learning and/or memory ability are impaired. Single transgenic mice were also tested and showed no significant difference comparing with wild-type control mice (Supplementary Material, Fig. S5).

Sociability and preference for social novelty are normal in I-Rai1 mice

To evaluate the sociability and the preference for social novelty behaviors of I-Rai1 mice, we utilized the three-chamber test (9,17). Briefly, the tested mice are allowed to freely explore in a three-chamber box with a central empty chamber, a chamber containing a stranger mouse (inside a corral) and a chamber with an empty corral. Wild-type, I-Rai1463 and I-Rai1479 all showed significant preference for the stimulus mouse, or stranger 1 mouse (wild-type $P = 0.003$, I-Rai1463 $P = 0.01$, I-Rai1479 $P = 0.025$) (Supplementary Material, Fig. S6A). We then put a new stranger mouse, stranger 2, into the previously empty corral to evaluate the preference for social novelty. They all showed the preference for the new stranger (wild-type: $P = 0.027$, I-Rai1463: $P = 0.03$, I-Rai1479: $P = 0.004$) (Supplementary Material, Fig. S6B), indicating that the mice present normal preference for social novelty.

Both lines were tested in our study and we found that they present the same phenotypes, lower body weight, hyperactivity, impaired learning and memory ability. Therefore, in the following Dox treatment experiments, we only utilized I-Rai1479.

Neurobehavioral defects in I-Rai1 mice can only be rescued by correcting the Rail dosage during development

In order to study the temporal relevance of Rail overexpression, we first set up the conditions for turning off the transgene. Two milligram per milliliter of Dox was added to the animals’ drinking water and we found that 2 weeks of Dox administration was enough to turn off the transgene expression (Supplementary Material, Fig. S7). To test if the phenotypes observed in I-Rai1 mice were a direct consequence of Rail overexpression, we treated them with Dox, in order to suppress the overexpression of Rail, from conception to 3 months of age (Fig. 4A). As expected, most of the phenotypes were absent in the I-Rai1 mice when the overexpression of Rail was abolished. Body weight of I-Rai1 mice at 4 months of age was not significantly different than their wild-type littermates (I-Rai1 mice: 29.51 ± 1.52 g versus WT mice: 32.49 ± 0.79 g, $P = 0.13$) (Fig. 4B). Abdominal fat weight was measured at 4 months of age and no significant difference was observed (I-Rai1 mice: 0.60 ± 0.14 g versus WT mice: 0.83 ± 0.09 g, $P = 0.22$) (Fig. 4C). Total distance travelled by the I-Rai1 mice was 6940 ± 778 cm, not significantly different when compared with the wild-type littermates (5544 ± 865 cm, $P = 0.25$) (Fig. 4D). Impaired contextual memory was also rescued in I-Rai1 mice (I-Rai1 mice: 47.99 ± 7.20% versus WT mice: 56.07 ± 6.22%, $P = 0.43$). The only exception was that the I-Rai1 mice still showed impaired memory ability in sound cue test (I-Rai1 mice: 10.06 ± 2.49% versus WT mice: 30.92 ± 7.86%, $P = 0.01$) (Fig. 4E). As sound cue memory was found normal in Dp(11)17/+ mice (7), most probably Rail dosage is not related to sound cue memory ability.

To test if the I-Rai1 mice neurons can restore normal function by correcting the Rail dosage, we performed the ‘prevention test’ (to turn off the transgene expression prior to the development of the phenotypes) and the ‘reversal test’ (to turn off the transgene expression after the development of the phenotypes).

In the ‘prevention test’, we turned off the transgene expression by Dox administration to the I-Rai1 mice and their wild-type littermates through the drinking water before the onset of the phenotypes (from 1 month of age to 3 months of age) (Fig. 5A). Surprisingly, all the phenotypes were still observed in the I-Rai1 mice. I-Rai1 mice started to show significant underweight from age 9 weeks (I-Rai1 mice: 21.72 ± 1.25 g versus WT mice: 25.85 ± 1.26 g, $P = 0.04$) (Fig. 5B). Hyperactivity was also observed in open field test (I-Rai1 mice: 12.397 ± 2035 cm versus wild-type mice: 6114 ± 1118 cm, $P = 0.03$) (Fig. 5C). In fear-conditioning test, I-Rai1 still showed impaired learning and memory abilities: in context test, I-Rai1 had significantly less percentage of freezing time (I-Rai1 mice: 22.25 ± 5.24% versus wild-type: 44.65 ± 6.78%, $P = 0.02$) and in sound cue test too (I-Rai1 mice: 9.68 ± 1.80% versus wild-type: 20.85 ± 5.27%, $P = 0.028$) (Fig. 5D). These data suggest that

### Table 1. General characterization of I-Rai1 mice and wild-type littermates

<table>
<thead>
<tr>
<th>Physical characteristics</th>
<th>I-Rai1463</th>
<th>I-Rai1479</th>
<th>Wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor coat condition (%)</td>
<td>8</td>
<td>0</td>
<td>11.1</td>
</tr>
<tr>
<td>Full whiskers (%)</td>
<td>100</td>
<td>100</td>
<td>90.3</td>
</tr>
<tr>
<td>Piloerection (%)</td>
<td>0</td>
<td>0</td>
<td>5.6</td>
</tr>
<tr>
<td>Neurological reflexes</td>
<td>100</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>Vocalization during handling (%)</td>
<td>17</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>Strength meter (Newton)</td>
<td>1.7</td>
<td>1.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Two feet</td>
<td>1.3</td>
<td>0.89</td>
<td>0.9</td>
</tr>
<tr>
<td>Four feet</td>
<td>1.7</td>
<td>1.8</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Evaluation of general characteristics and reflexes was performed in $N = 35$ wild-type, $N = 12$ I-Rai1463 and $N = 12$ I-Rai1479 male mice at 3 months of age.
the development of the phenotypic manifestations is not depend-
ent on the presence of overexpressed \textit{Rai1} at the time of the ob-
servable phenotypic onset, but it is most likely the result of
abnormal development. Next, we did the ‘reversal test’ to deter-
mine whether the phenotypes that were developed in the pres-
ence of \textit{Rai1} overexpression required continuous overexpression
for their expressivity by turning off the transgene expression
after the onset of the PTLS phenotypes (from 3 to 5 months of
age) (Fig. 5E). I-Rai1 mice did not show any signs of recovery
after 2 months of Dox treatment. Before Dox treatment, I-Rai1
mice already showed significant underweight at 3 months of
age (I-Rai1 mice: 23.22 ± 2.14 g versus wild-type mice:
30.16 ± 0.85 g, P = 0.017) (Fig. 5F) and during the 2 months of Dox administration, their body weight continued to be significantly underweight (Fig. 5G). At 6 months of age, I-Rai1 mice showed significantly less abdominal fat (I-Rai1 mice: 0.31 ± 0.04 g versus wild-type mice: 0.57 ± 0.08 g, P = 0.04). Hyperactivity was also observed in open field test (I-Rai1 mice: 6503 ± 509 cm versus wild-type mice: 3988 ± 402 cm, P = 0.006) (Fig. 5H). The fear-conditioning test did not show recovery either in the context test. I-Rai1 had significantly less percentage of freezing time (I-Rai1 mice: 11.66 ± 3.27% versus wild-type mice: 58.69 ± 9.46%, P = 0.002) and in the sound cue test too (I-Rai1 mice: 10.43 ± 2.89% versus wild-type mice: 29.15 ± 7.02%, P = 0.038) (Fig. 5I). Importantly, in order to show that the transgene was turned off successfully, at the end of each experiment we obtained brain sections from all the I-Rai1 mice treated with Dox and eGFP fluorescence was analyzed in brain slides. No transgene expression was observed in the confocal images for any of the Dox-treated mice (Supplementary Material, Fig. S8).

In conclusion, our results suggest that Rai1 overexpression during development in mice is enough to the development of PTLS phenotypes in I-Rai1 mice, and after development, the damages caused are not reversible by correcting Rai1 gene expression levels.
Figure 5. Dox administration from 1 to 3 months did not prevent the development of PTLS-like phenotypes and Dox treatment from 3 to 5 months did not rescue the PTLS-like phenotypes. (A) Diagram showed Dox administration time period and the starting time of behavior tests for prevention test (WT: n = 6 I-Rai1: n = 8). (B) Weight curves for wild-type (WT) and I-Rai1 male mice with Dox administration from 4 to 12 weeks. (C) Locomotor activity of WT and I-Rai1 male mice with 1–3-month Dox administration in the open-field test. (D) The percentage of freezing time in fear-conditioning test (context and sound-cue) for WT and I-Rai1 male mice with Dox administration from 1 to 3 months. (E) Diagram showed Dox administration time period and the starting time of behavior tests for the reversal test (n = 5 for each genotype). (F) Weight curves for WT and I-Rai1 male mice with Dox administration from 3 to 5 months. (G) Abdominal fat at 6 months of age from WT and I-Rai1 male mice with Dox administration was weighed, and each bar represents the average of results for five samples. (H) Locomotor activity of WT and I-Rai1 male mice with 3–5-month Dox administration in the open-field test. (I) Percentages of freezing time in fear-conditioning test (context and sound-cue) for WT and I-Rai1 male mice with Dox administration from 3 to 5 months are shown. Values represent mean ± SEM. The asterisk denotes significantly different (*P < 0.05, **P < 0.01). White bars: WT; Black bars: I-Rai1.
DISCUSSION

PTLS is a neurodevelopmental disorder mainly caused by the duplication of the RAI1 gene. The Dp(11)17/+ mouse model generated previously recapitulates important neurobehavioral phenotypes observed in PTLS patients, indicating PTLS phenotypes can be reproduced in mice. Here, using a doxycycline-responsive gene system, we have demonstrated that forebrain neuron overexpression of Rail is enough to cause PTLS-like phenotypes in a mouse model and that Rail overexpression during development is sufficient to the occurrence of these phenotypes. In our study, we generated and studied two lines of I-Rai1 to control for possible position effect of the transgene. Both lines of I-Rai1 mice showed PTLS-like phenotypes such as underweight, hyperactivity and learning and memory deficits, suggesting the importance of Rail dosage in neurodevelopment or normal neuron function. In addition, we employed doxycycline administration to turn off transgene expression at different stages of mouse development and postnatally and we found that shutting off the overexpression of Rail since embryonic development is necessary to prevent the development of PTLS-like phenotypes in I-Rai1 mice.

Decreased body weight is observed both in PTLS patients and the Dp(11)17/+ mice. Also, an independent PTLS mouse model, carrying a transgenic BAC-Rai1 presented growth retardation and reduced body weight (18). It was also shown that by correcting Rail dosage, the leanness phenotype in Dp(11)17/+ mice can be rescued (8). All these evidences indicated that Rail is a regulator of body weight. Here, we found that the main effect of Rail for the regulation of body weight and fat content is located in forebrain neurons. In recent years, many studies focus on the role of hippocampus in energy balance and linked the function of hippocampus in learning and memory with energy regulation (19).

Therefore, overexpression of Rail in hippocampus might also be responsible for the body weight changes since the transgene has very low expression in hypothalamus of I-Rai1 mice.

Hyperactivity was previously reported for PTLS patients and Dp(11)17/+ mice, and it was shown that both genomic structural changes and Rail dosage contributes to hyperactivity (8,20). We here demonstrate that elevated Rail dosage in forebrain neurons during development is enough to cause increased activity.

Learning difficulties were observed in I-Rai1 mice as evidenced by reduced freezing in the fear-conditioning test. PTLS patients present mild-to-moderate intellectual disabilities and autism (1,2,6). In addition, the PTLS mouse model has impairment in learning and memory (7,8,18) and abnormalities in social behaviors (9,20). In fear conditioning, I-Rai1 mice showed both contextual and sound-cue memory defect and only contextual defect is rescued by turning off transgene expression during development. It was reported that in Dp(11)17/+ mice, a trend of less freezing was found in the sound-cue test without significant differences (7). These results indicated that Rail dosage during development is directly linked with contextual defect observed in I-Rai1 mice, whereas Rail dosage is not the cause of sound-cue memory defect in I-Rai1 mice. Sound-cue memory seems to be rather a complicated process and the defect observed in I-Rai1 might be due to the position effect of the compound double transgenic mice. Thus I-Rai1 is a good model to study the relationship between Rail dosage and impaired memory and learning ability.

Anxiety-like behavior and abnormalities in social behaviors were observed in the PTLS mouse model Dp(11)17/++; however, Rail dosage alone might not be responsible for the anxiety-like behavior and abnormal social behaviors observed in the PTLS mouse model Dp(11)17/+. In previous study, by crossing the SMS mouse model Df(11)17/+ and PTLS mouse model Dp(11)17/+, in Df(11)17/Dp(11)17 mice, although Rail gene dosage is corrected, complex behaviors such as anxiety and preference for social novelty were still abnormal, indicating that genomic alterations might be involved in these complex behaviors (20). Our results demonstrate that Rail dosage alteration alone in forebrain neurons is not sufficient to cause anxiety. A recent study showed that PTLS patients with a short duplication (<1 Mb) containing mainly RAI1 gene also present autistic features (5), indicating both genomic structural change and Rail dosage alteration contribute to abnormal social behavior. In our study, no significant social novelty preference differences were found between I-Rai1 mice and wild-type mice, which might be because the overexpression of Rail in other brain regions is required for the social abnormalities.

For example, a previous study reported that the absence of integrin β3 which interacts with serotonin transporter in midbrain cause abnormal social novelty behavior (21). The behavior phenotypes found in this study are summarized in Table 2, together with phenotypes reported previously for Dp(11)17/+ mice. Our mouse model replicated several of the phenotypes observed in Dp(11)17/+ mice.

Rail dosage seems to be essentially important during neuronal development. In some genetic conditions such as Rett syndrome and Huntington’s disease, it was shown that the continuous expression of mutant protein or dosage imbalance is required to maintain the symptoms (22,23). In our study, by turning off Rail transgene expression from 1 to 3 months or 3 to 5 months, we can neither prevent nor reverse the development of the PTLS-like phenotypes observed in I-Rai1 mice. In order to rescue the phenotypes, correct Rail dosage is required during embryonic and/or first postnatal month development, suggesting that developmental overexpression of Rail lead to damage not reversible by normalization of Rail expression and the development of PTLS-like phenotypes in our system. Thus, we support the notion of PTLS being a neurodevelopmental syndrome. Our results suggest that drugs aimed to down-regulate Rail expression may not work for PTLS patients because they have already passed the developmental stage at which normal Rail dosage is required. However, as proved in mice already (24), alternatives such as environment enrichment could be utilized to ameliorate or even rescue the observed neurobehavioral abnormalities. Caution should be taken when we extrapolate these results and compare with human patients, since it is recognized that mouse models do not always mimic the corresponding human conditions. These differences between mouse and human should be taken into account especially when using mice as preclinical models. In addition, I-Rai1 is a mouse model only overexpressing Rail in forebrain neurons which might create potential differences.

In summary, our study shows that Rail dosage in forebrain neurons during development in mice is crucial in body weight regulation, activity and learning and memory abilities. Therefore, we provided a specific mouse model to study Rail function at specific development stage and to understand the pathogenesis of PTLS.
Table 2. Phenotypic comparison of PTLS mouse models

<table>
<thead>
<tr>
<th>Genotype Phenotype</th>
<th>Dp(11)17/+</th>
<th>I-Rai1</th>
<th>I-Rai2</th>
<th>I-Rai3</th>
<th>I-Rai4</th>
<th>I-Rai5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Body weight</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Abdominal fat</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Brain weight</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Vocalization while handling</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Anxiety levels</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Fear conditioning (contextual)</td>
<td>Impaired</td>
<td>Impaired</td>
<td>Impaired</td>
<td>Normal</td>
<td>Impaired</td>
<td>Impaired</td>
</tr>
<tr>
<td>Fear conditioning (Sound cue)</td>
<td>Normal</td>
<td>Impaired</td>
<td>Impaired</td>
<td>Normal</td>
<td>Impaired</td>
<td>Impaired</td>
</tr>
<tr>
<td>Activity in open field</td>
<td>Hyperactive</td>
<td>Hyperactive</td>
<td>Hyperactive</td>
<td>Normal</td>
<td>Hyperactive</td>
<td>Hyperactive</td>
</tr>
<tr>
<td>Strength meter</td>
<td>Normal‡</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Sociability</td>
<td>Normal§</td>
<td>Normal</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Preference for social novelty</td>
<td>Decreased</td>
<td>Normal</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

A summary of the phenotypes that were found abnormal in Dp(11)17/+ mice and in I-Rai1 mice.
N/A: data not available.
1I-Rai1 without Dox administration, both lines I-Rai1<sup>143</sup> and I-Rai1<sup>179</sup> showed essentially the same phenotypes.
2<sup>Waliz et al</sup> (7), Molina et al (9).
3Abnormal sociability and decreased brain weight were reported in Dp(11)17/+ mice in a pure C57BL/6-Tyr-<sup>Brd</sup> genetic background. Molina et al. (9).
4<sup>c</sup>d<sup>x</sup>-I-Rai<sup>179</sup> animals treated with Dox from conception to 3 months, 1–3 months and 3–5 months, respectively.
5Significantly different from their wild-type littermates, <i>P</i> < 0.05.

MATERIALS AND METHODS

Cloning of murine Rai1 cDNA into the pBi-eGFP vector

The murine Rai1-HA cDNA was obtained as described in Carmona-Mora <i>et al</i>. 2010. This cDNA was subcloned into the <i>Sha/Unhcl</i> multiple cloning site of the pBi-eGFP vector (Clontech). The sequence of Rai1-HA-pBi-eGFP was confirmed by direct sequencing.

In vitro studies

To test the Rai1-HA-pBi-eGFP/pTet-off system, we performed western blot and immunofluorescence analysis. Neuro2A cells were transfected with 4.0 µg of the corresponding plasmids using Lipofectamine2000 (Invitrogen) in six-well plate. Twenty-four hours post-transfection, cells were lysed in Blue buffer (2% SDS, 2 m urea, 10% Glycerol, 10 mM Tris pH 6.8, 0.02% Bromophenol Blue and 10 mM DTT) containing 1× proteases inhibitors cocktail (Sigma). Twenty microfilters of total protein from each condition were run in a 10% SDS–PAGE gel, transferred to a 0.2 µm PVDF membrane, blocked with 5% not fat milk in TTBS (137 mM NaCl, 0.1% Tween 20 and 20 mM TrisHCl, pH 7.6) and incubated with a rabbit anti-HA 1:8000 (ab47679, Abcam) overnight at 4°C. After three washes of 30 min each with TTBS, the membrane was incubated with a HRP-conjugated goat anti-rabbit 1:8000 (Pierce) for 2 h. Western blots were then developed using Pierce Super Signal West Dura Extended Duration Substrate. Neuro2a cells were transfected with pTet-off and Rai1-HA-pBi-eGFP separately or together (with or without the treatment of Doxycycline). Cells were treated with Doxycycline (324 385, Calbiochem) 6 h after transfection for the final concentration of 10 ng/ml. All transfections were performed using Lipofectamine2000 as described above. The cells were fixed 24 h post-transfection with PFA 4%. Afterwards, cells were treated with PBS-TX-100 0.1% for 15 min and blocked with PBS-gelatin 0.2% for 1 h. Next, we incubated with a rabbit anti-HA 1/1000 (BETHYL) antibody and with a goat anti rabbit Alexa Fluor 568 (Invitrogen). Mounting was done with DAPI dye. Images were captured with a LSM710 Zeiss confocal microscope.

Generation and molecular characterization of transgenic mice

The transgene construct Rai1-HA-pBi-eGFP was digested by <i>Ase1</i> and <i>Pvu1</i> to eliminate the bacterial components and linearize the mammalian expression components. The purified linear fragment was introduced into pronuclei of C57B6/6T CBA/J zygotes. The transgenic mice were generated at the Centro de Estudios Científicos (CECs), in Valdivia, Chile. Genotyping of the progeny was performed by PCR and Southern blot and three founders were selected. To generate conditional Rai1 over-expressing mice, Rai1-HA-pBi-eGFP (or pBi-Rai1) transgenic mice were crossed with Camk2-tTA mouse line [B6;CBA-TgN(Camk2Ta)1Mmay, from Jackson Laboratories] (15). The mouse lines are maintained in a mixed C57BL/6 background.

Mice were maintained in a SPF facility with a 12 h light-dark cycle with access to food and water <i>ad lib</i>. Mice were housed in groups of 2–5 per cage. Mice with doxycycline administration were given 2 mg/ml Dox in 5% sucrose solution instead of the regular drinking water. Dox solution was kept in dark bottles and changed once a week. All testing procedures were approved by University of Miami Animal care and use committee and followed NIH Guidelines.

Genotyping

Genomic DNA was isolated from 14 to 21 days old mice tails and routine genotyping was carried out using specific primers for pBi-Rai1 mice (CMV forward: 5′-GGCTGGTTTAGGACCCGT CAG-3′, Rai1 411 reverse: 5′-GTTCTCCTGATACTGCTCACTC
Real-time PCR
Samples from olfactory bulb, cortex and hippocampus were obtained from 6-month-old mice. Total RNA was isolated with TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. Prior to reverse transcription, RNA samples were treated with rDNase I (DNA-free kit, Applied Biosystems). Then cDNA was synthesized using Improm-II Reverse Transcriptase System (Promega). We eliminated the possibility of genomic DNA contamination by using rDNaseI and ‘no RT’ controls in the real-time PCR reactions. We performed quantitative real-time PCR with QUANTIMIX EASY SYG kit (BIO-TOOLS B&M Labs). The primers used for detecting Rai1 transcripts were forward 5'-CATGAATTGCAAGGAGGC-3′ and reverse 5'-GGTCTATTGCACTAGGTA-3′. Results were normalized against Cyclophilin A (forward 5'-GGGATTGCTGACAACCTAGG-3′ and reverse 5'-GCAATGCTGGACCAAA CCAA-3′). All reactions were performed in triplicate with the following amplification parameters: 10 min at 95°C, and 40 cycles of 10 s at 95°C, 30 s at 58°C and 30 s at 72°C. The delta Ct method was used to compare the DCt (cycle threshold) value of transgenic animal samples (Ct of target –Ct of control transcript) with the DCt value of wild-type mice samples.

In vivo protein expression analysis
For western blot, mouse brain was dissected and homogenized directly with SDS buffer (25 mM Tris-Cl pH 7.4, 25 mM sodium citrate, 2% SDS, 5 mM CaCl2, 0.5 mM DTT). Cell lysates were boiled at 70°C for 10 min followed by brief sonication to reduce cell lysis viscosity. Cell lysates were incubated with 4× sample buffer before use and loaded into precast gels. Rat anti-HA was used to detect transgene expression. Cell fractionation was essential according to reference (13). For immunofluorescence, mouse brain was dissected, rinsed with PBS for three times and then fixed in 4% PFA overnight. Forty micrometer sections were made with a vibrotome. The sections are incubated with DAPI for 10 min, rinsed with PBS for two times and mounted with the mounting medium. For Neuron and glial cell labeling, sections were blocked with blocking buffer (5% BSA, 0.01% Triton-X100) and incubated with primary antibodies (Rabbit anti-GFAP 1:1000, Abcam Ab7260; mouse anti-NeuN 1:1000, Chemicon MAB377). Goat anti-mouse Alexa Fluor568 and Goat anti-rabbit Alexa Fluor568 (Invitrogen) were used as secondary antibodies. Immunofluorescence pictures were taken using a confocal microscope (LSM710 Zeiss).

Primary neuronal culture and image analysis
Hippocampal and cortical cells were cultured as previously described (25). Briefly, hippocampus and cortex were dissected from newborn mice brains and dissociated cells were plating on poly-l-lysine coated cover slips. At 3 days in vitro (DIV), Cells were fixed and stained with Anti-HA 1/1000 (Roche) antibody and with a goat anti-rat Alexa Fluor 568 (Invitrogen). The slides were then incubated with DAPI and mounted. Images were captured with a LSM710 Zeiss confocal microscope.

Mouse phenotypic characterization
At 3 months of age, mouse behaviors were tested in the following order with 0–2 days between each test: (i) general health and neurological reflexes, (ii) open field, (iii) plus maze, (iv) three-chamber test, and (v) fear-conditioning test. Mouse numbers used in the test are as follows: initial phenotypic characterization without Dox administration (n ≥ 8 for each genotype); Dox treatment from conception to 3 months (n ≥ 8 for each genotype); Dox treatment from 1 to 3 months (WT: n = 6, I-Rai1: n = 8); Dox treatment from 3 to 5 months (WT: n = 5, I-Rai1: n = 5).

General characterization
At the beginning of the behavioral battery, general health of each mouse was evaluated including mouse fur and whisker condition, piloerection, visual placing reflex, vocalization and vibrissae orienting. The forelimbs and four feet strength were measured by a grip strength meter.

Open field
The open-field test was used to assess exploratory activity and anxiety-related responses in a novel arena. The mouse was placed in the center of a clear Plexiglas (40 cm × 40 cm × 30 cm) open-field arena and allowed to explore for 30 min. Activity in the open field is quantitated by a computer-operated activity monitor program from the Med Associates system.

Elevated plus maze
Elevated plus maze was used to evaluate mouse anxiety. The elevated plus maze consists of two closed arms with 20 cm height walls, and two open arms. Each arm is 33 cm long and the plus maze is elevated 50 cm from the floor. Mice were transferred into the behavior room 30 min before the test. A mouse was placed in the center of the maze and allowed to freely explore the apparatus for 5 min video and data were recorded and analyzed with Med Associates software.

Fear conditioning test
Starting around 10:30 am, the mice were placed in the operant chambers and underwent a 3 min pre-context test of fear conditioning. The mice were taken out and put back immediately for the fear-conditioning training. After a 3 min acclimation period, the mouse received two pairings of a 30 s tone (2.8 kHz, 80 db) followed by a footshock unconditioned stimulus (US; 2 s, 0.75 mA). The mice were returned to their home cages for 2 h, and then they were returned to the chambers to test their contextual memory ability. They were returned to their home cage and after 1 h, they were placed in the same chamber with changed context and odor. Thirty-second tone without footshock was presented to test their sound-cue memory ability. Mouse freeze software (MedPC-IV; Med Associates) was used to analyze their freeze time during the test.
Organisms weight measurements

Different organs from the mice were sacrificed immediately after the mice were sacrificed including cerebellum, spleen, heart, kidney and abdominal fat pads (visceral adipose tissue). The weights were recorded.

Statistical analysis

All the data were analyzed by Student’s t-test and P < 0.05 was considered significant. Chi-square was used to analyze vocalization during handling difference between different genotypes. Values are displayed as mean ± standard error of mean (SEM).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Cesar P. Canales, Irene C. Perez, and Vladimir Camarena for providing assistance and advice in this study and Yaima Toledo Fernandez and Karen Neagley for the English proofreading and formatting of the manuscript.

Conflict of Interest statement. None declared.

FUNDING

This study was supported in part by The Jerome Lejeune Foundation Grant to K.W. and by FONDECYT, Chile (grant 1061067 to K.W.). The Transgenic Facility at CECs is supported by the Basal Financing program of CONICYT, Chile.

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