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### Authors

Patriarchi, Tommaso  
Amabile, Sonia  
Frullanti, Elisa  
[et al.](#)

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ARTICLE

# Imbalance of excitatory/inhibitory synaptic protein expression in iPSC-derived neurons from *FOXG1*<sup>+/-</sup> patients and in *foxg1*<sup>+/-</sup> mice

Tommaso Patriarchi<sup>1,2</sup>, Sonia Amabile<sup>1</sup>, Elisa Frullanti<sup>3</sup>, Elisa Landucci<sup>1</sup>, Caterina Lo Rizzo<sup>1</sup>, Francesca Ariani<sup>1,3</sup>, Mario Costa<sup>4,5</sup>, Francesco Olimpico<sup>4</sup>, Johannes W Hell<sup>2</sup>, Flora M Vaccarino<sup>6,7</sup>, Alessandra Renieri<sup>\*,1,3</sup> and Ilaria Meloni<sup>1</sup>

Rett syndrome (RTT) is a severe neurodevelopmental disorder associated with mutations in either *MECP2*, *CDKL5* or *FOXG1*. The precise molecular mechanisms that lead to the pathogenesis of RTT have yet to be elucidated. We recently reported that expression of GluD1 (orphan glutamate receptor  $\delta$ -1 subunit) is increased in iPSC-derived neurons obtained from patients with mutations in either *MECP2* or *CDKL5*. GluD1 controls synaptic differentiation and shifts the balance between excitatory and inhibitory synapses toward the latter. Thus, an increase in GluD1 might be a critical factor in the etiology of RTT by affecting the excitatory/inhibitory balance in the developing brain. To test this hypothesis, we generated iPSC-derived neurons from *FOXG1*<sup>+/-</sup> patients. We analyzed mRNA and protein levels of GluD1 together with key markers of excitatory and inhibitory synapses in these iPSC-derived neurons and in *Foxg1*<sup>+/-</sup> mouse fetal (E11.5) and adult (P70) brains. We found strong correlation between iPSC-derived neurons and fetal mouse brains, where GluD1 and inhibitory synaptic markers (GAD67 and GABA AR- $\alpha$ 1) were increased, whereas the levels of a number of excitatory synaptic markers (VGLUT1, GluA1, GluN1 and PSD-95) were decreased. In adult mice, GluD1 was decreased along with all GABAergic and glutamatergic markers. Our findings further the understanding of the etiology of RTT by introducing a new pathological event occurring in the brain of *FOXG1*<sup>+/-</sup> patients during embryonic development and its time-dependent shift toward a general decrease in brain synapses.

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## INTRODUCTION

Rett syndrome (RTT, OMIM #312750) is a severe neurodevelopmental disorder that affects ~1 in 10 000 females. It is characterized by cognitive impairment, autism-like features, stereotypic hand movements, hypotonia, microcephaly and often epilepsy.<sup>1</sup> Although classic RTT is due to mutations in *MECP2*, variants of this disease are caused by mutations in *CDKL5* and *FOXG1*.<sup>2,3</sup> In particular, mutations in *FOXG1* give rise to the congenital variant that shows classic RTT features but earlier onset (first months of life).<sup>4,5</sup>

*FOXG1* (also known as *XBF-1*) is a member of the winged-helix gene family and is widely expressed in the forebrain at all developmental stages and in adulthood. It functions as a transcriptional repressor and is fundamental for normal brain development.<sup>6,7</sup> The generation of *Foxg1* knockout mice showed that this gene is essential for survival, as *Foxg1*<sup>-/-</sup> animals die at birth and have a dramatic reduction in the size of brain hemispheres.<sup>8</sup> Heterozygous *Foxg1*<sup>+/-</sup> mice show remarkable alterations in their hippocampal structures and contextual memory deficits.<sup>6,9</sup> Numerous studies have shown that *FOXG1* is an important neurodevelopmental factor, in particular for the telencephalon, where it is involved in the timing and specification of the neuronal fate and essential for postmitotic neuronal survival.<sup>10–13</sup>

The level of FoxG1 is tightly controlled. In fact, both heterozygous *FOXG1* duplication and deletion are associated with severe cognitive impairment, loss of speech and developmental epilepsy.<sup>14,15</sup> *FOXG1* overexpression inhibits gliogenesis and promotes neurogenesis in neural stem cells (NSCs); in fact, it can reprogram fibroblasts into neural precursor-like cells when paired with *PAX6* overexpression.<sup>16,17</sup> On top of this, FoxG1 directly controls the levels of *PAX6*, another transcription factor equally important for promoting NSC proliferation and inhibiting premature neuronal differentiation during early forebrain development.<sup>18–25</sup>

The clinical overlap of symptoms between *MECP2*-, *CDKL5*- and *FOXG1*-mutated RTT patients raises the possibility that they share a common pathological mechanism. By studying patient-specific iPSC-derived neurons with mutations in *MECP2* or *CDKL5*, we recently identified *GRID1* (coding for GluD1, orphan glutamate receptor  $\delta$ -1 subunit) as a common deregulated gene.<sup>26</sup> Both mRNA and protein levels of *GRID1* are increased in mutated *versus* control neurons. GluD1 shares homology with the other members of the ionotropic glutamate receptor family but, in contrast to those, it does not appear to form receptors that are able to conduct ions. Instead, it is emerging as a synaptic cell adhesion protein that can induce formation of synaptic structures.<sup>27</sup> Recent evidence now indicates that GluD1 can induce preferentially the formation of inhibitory GABAergic synapses

<sup>1</sup>Medical Genetics, University of Siena, Siena, Italy; <sup>2</sup>Department of Pharmacology, University of California, Davis, Davis, CA, USA; <sup>3</sup>Genetica Medica, Azienda Ospedaliera Universitaria Senese, Siena, Italy; <sup>4</sup>Institute of Neuroscience, Italian National Research Council, Pisa, Italy; <sup>5</sup>Scuola Normale Superiore, Pisa, Italy; <sup>6</sup>Child Study Center, Yale University School of Medicine, New Haven, CT, USA; <sup>7</sup>Department of Neurobiology, Yale University School of Medicine, New Haven, CT, USA  
\*Correspondence: Professor A Renieri, Medical Genetics, Department of Medical Biotechnologies, University of Siena, Viale Bracci 2, S. Maria alle Scotte, Siena, Italy. Tel: +39 577 233303; Fax: +39 577 233325; E-mail: alessandra.renieri@unisi.it

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by interacting with presynaptic neurexin in conjunction with cerebellin.<sup>28,29</sup> Another study directly links GluD1 to the formation and maintenance of the excitatory synapses between parallel fibers (PFs) and interneurons in the cerebellum that ultimately promotes the survival of the inhibitory interneurons. In fact, GluD1<sup>-/-</sup> mice show a marked reduction (35%) in the numbers of these inhibitory neurons in the cerebellum.<sup>30</sup> In addition, genetic variation in the *GRID1* promoter is strongly associated with schizophrenia and autism.<sup>31,32</sup> The increase in GluD1 levels observed in iPSC-derived RTT neurons could therefore lead to an excitatory/inhibitory (E/I) imbalance in synaptic or neuronal differentiation that may underlie RTT symptoms such as cognitive impairment and autistic features. This possibility seems even more likely as a gain-of-function mutation in another transsynaptic adhesion molecule that acts similarly to GluD1 in synapse differentiation, neuroligin 3 (NLG3), is associated with autism spectrum disorders (ASDs).<sup>33,34</sup> Neuroligin 3 is another postsynaptic partner for presynaptic neurexins at excitatory and inhibitory synapses.<sup>35</sup> Transgenic mice carrying the same gain-of-function mutation in *Nlg3* as ASD patients have impaired social interactions and increased inhibitory synaptic transmission but normal excitatory transmission.<sup>34</sup> Hence, we hypothesize that an E/I shift toward inhibition occurring during early embryonic stages contributes to the etiology of RTT. To determine whether GluD1 is upregulated in *FOXG1*<sup>+/-</sup> neurons and whether this corresponds to increased inhibition, we generated for the first time iPSCs from *FOXG1*<sup>+/-</sup> patients and differentiated them into neurons. Using this patient-derived human cellular model and a *Foxg1*<sup>+/-</sup> mouse model, we analyzed both gene expression and protein levels of *GRID1* together with key excitatory and inhibitory synaptic markers.

## MATERIALS AND METHODS

### Generation and maintenance of *FOXG1*<sup>+/-</sup> iPSC cells

The iPSCs were generated from fibroblasts of two patients referred to our laboratory for genetic counseling and molecular diagnosis (<http://www.biobank.unisi.it/Elencorett.asp>; patient codes 146 and 361, respectively). Following informed consent signature, skin biopsies (~3–4 mm<sup>3</sup>) were performed using the Punch Biopsy procedure. Fibroblasts were isolated and cultured according to standard protocols.<sup>36</sup> Cells at low passages (2–3) were used for reprogramming following the procedure by Takahashi *et al.*<sup>37</sup> using the four Yamanaka retroviral vectors. The iPSCs were cultured on matrigel-coated (BD Biosciences, Milano, Italy) cell culture plates in mTeSR1 medium (Stem Cell Technologies, Grenoble, France), supplemented with 50 U/ml penicillin and 50 mg/ml streptomycin. Cells were passed once every week using Dispase (Stem Cell Technologies).

### Neuronal differentiation of iPSCs

Neuronal differentiation was accomplished with a protocol that preferentially directs the differentiation of iPSCs into forebrain neural progenitors.<sup>38</sup> Briefly, iPSCs were detached from the culture plate and forced to aggregate into EB-like structures in Aggrewell plates (Stem Cell Technologies) with a medium containing Noggin and DKK (200 ng/ml each) for 2 days. The cell aggregates were then grown in suspension for 2 additional days in the same medium and then allowed to attach onto matrigel for the formation of neuronal rosettes, consisting of neuroepithelial cells arranged in a tubular structure. Rosettes were manually picked, dissociated and expanded for isolation of neuronal progenitors or subsequent terminal differentiation. In spite of the presence of Noggin that should direct differentiation toward a forebrain fate, we obtained heterogeneous cultures containing a variable percentage of nonneuronal cells. Neuronal progenitors and neuronal cells were isolated for quantitative analyses by immunomagnetic cell sorting with magnetic beads-conjugated antibodies against PSA-NCAM and CD24, respectively (Miltenyi Biotec, Calderara di Reno, Bologna, Italy).<sup>26,39</sup>

## Immunofluorescence

The detailed protocol used for immunofluorescence staining of our cellular cultures is provided in the Supplementary Materials.

## Real-time qRT-PCR

The mRNA was extracted from iPSC-derived neuronal progenitors and neurons with RNeasy mini kit (Qiagen, Milano, Italy). Quantitation was performed using commercial TaqMan probes from Applied Biosystem (Life Technologies, Monza, Italy) (*GRID1* assay ID: Hs00324946\_m1; *GRIN1* assay ID: Hs00609561\_m1; *GRIA1* assay ID: Hs00990737\_m1; *VGLUT1*: Hs00220404\_m1; *VGLUT2* assay ID: Hs00220439\_m1; *GAD1* assay ID: Hs01065893\_m1). The cyclophilin (*CYCLO*) gene was used as a reference (assay ID: Hs01565700\_g1). Student's *t*-test with a significance level of 95% was used for the identification of statistically significant differences in expression levels among different conditions.

## *Foxg1*<sup>+/-</sup> mice

Animals were housed in a 12-h light/dark cycle with free access to food and water. All *Foxg1*<sup>+/-</sup> mice used in this study were generated by heterozygous/wild-type matings of the original *Foxg1*-cre background (C57BL/6J).<sup>40</sup> C57BL/6J (wild type) mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). The 11.5 day-pregnant females were killed by cervical dislocation. Forebrain regions of embryos aged E11.5 were carefully dissected (under dissection microscope). Adult mice were anesthetized with isoflurane before cervical dislocation. All brain tissues were immersed in a buffer containing 0.01 M Tris/HCl, 1 mM EGTA, 0.2 M sucrose and protease inhibitors before being snap-frozen. All procedures were carried out in accordance with the directives of the European Community Council (86/609/EEC) and approved by the Italian Ministry of Health.

## Immunoblotting

Proteins from iPSC-derived neurons were extracted with 10-fold excess of RIPA buffer. For protein extraction from mouse brains a Triton X-100-based lysis buffer was used (see Supplementary Materials). Protease inhibitor cocktail (Sigma, Milano, Italy) was added to all lysates. Lysates were cleared by ultracentrifugation (200 000 g for 30 min at 4 °C) before western blot analysis.

## Statistical analysis

All data are presented as mean ± SEM. The iPSC lines analyzed in this study were generated from a total of two patients for both control and *FOXG1*<sup>+/-</sup> condition. The number of iPSC lines analyzed for each condition is reported as *n* in the bar graphs. For all statistical analyses, *P*-value was determined with Student's *t*-test using Graph Pad Prism 5.0 software (San Diego, CA, USA).

## RESULTS

### Generation and characterization of *FOXG1*<sup>+/-</sup> iPSC cells

The iPSC lines used in this study were generated from two female patients with mutations in *FOXG1* (NM\_005249.4). Patient 1 carrying c.765G>A (p.(Trp255\*)) mutation has been previously described.<sup>2</sup> Patient 2 carried a genomic deletion (hg19 chr14: g.28552714\_29655318del) including the entire *FOXG1* gene and a locus that is transcribed into a noncoding RNA of unknown function (C14orf23) (Decipher ID #314441). Clinical phenotype together with the clinical severity scores of both patients is reported in Supplementary Table S1. The newly reprogrammed cells assumed the correct morphology (Supplementary Figure S1A), correctly induced the expression of endogenous pluripotency genes (*OCT4*, *NANOG*, *DNMT1* and *SOX2*) and silenced the virally transduced ones, as assessed by real time RT-PCR with the use of freshly transduced fibroblasts as positive control (Supplementary Figures S1B and C). All iPSC clones maintained normal molecular karyotype. Direct sequencing (patient 1) and array-CGH (patient 2) demonstrates

the expected *FOXG1* alterations, confirming that clones were derived from respective fibroblasts (Supplementary Figures S2 and S3).

Two clones from patient 1 (ID 156#14 and #16) and two clones from patient 2 (ID 2362#5 and #9) were selected for subsequent analyses. For comparison we used iPSCs derived from a normal male (BJ; control 1) and clones from two different female *MECP2*-mutated patients (ID 143#14 and 2271#2; controls 2 and 3) who showed exclusive expression of the wild-type *MECP2* allele because of inactivation of the X chromosome with the mutant *MECP2* (Supplementary Figure S4).

### Neuronal differentiation from iPSCs

Neuronal differentiation of *FOXG1*-mutated iPSCs was performed using a published protocol that allows directing the differentiation of iPSCs into forebrain neural progenitors<sup>38</sup> (Figure 1a). As expected, the neurons obtained with this protocol are mainly glutamatergic (positive staining for vesicular glutamate transporter 1 (VGLUT1)), although some GABAergic neurons (positive staining for GAD65/67) are also present (Figure 1b). The protocol results in heterogeneous cultures containing a variable percentage of nonneuronal cells. In order to isolate a pure population of neuronal progenitors and neurons for subsequent quantitative analyses, we thus employed an immunomagnetic cell sorting protocol based on the use of anti-PSA-NCAM (neuronal progenitors) or anti-CD24 (neurons) antibodies conjugated to magnetic beads.<sup>26,39</sup>

### Increased GluD1 expression

We recently demonstrated that GluD1, which is encoded by *GRID1* gene, is significantly overexpressed in neuronal precursors and mature neurons from patients with mutations in both *MECP2* and *CDKL5*.<sup>26</sup> We thus performed in-depth analysis of GluD1 expression in *FOXG1*-mutant cells. The mRNA levels for *GRID1* showed a statistically significant twofold increase in *FOXG1*<sup>+/-</sup> neurons (Figure 2a,  $P < 0.05$ ). A statistically significant increase of GluD1 expression was

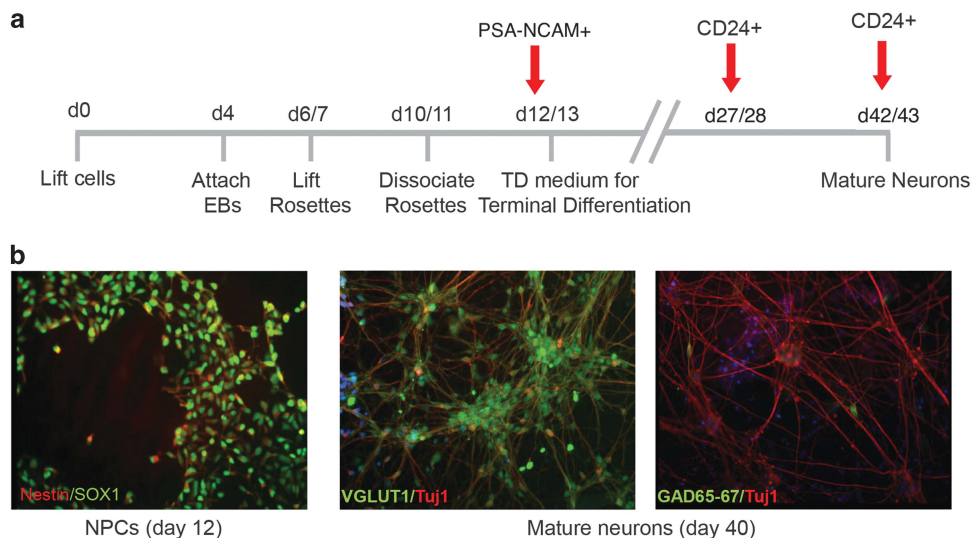
also seen at the protein level within these neurons (Figure 2b,  $P < 0.01$ ).

### Excitatory/inhibitory synaptic markers unbalance

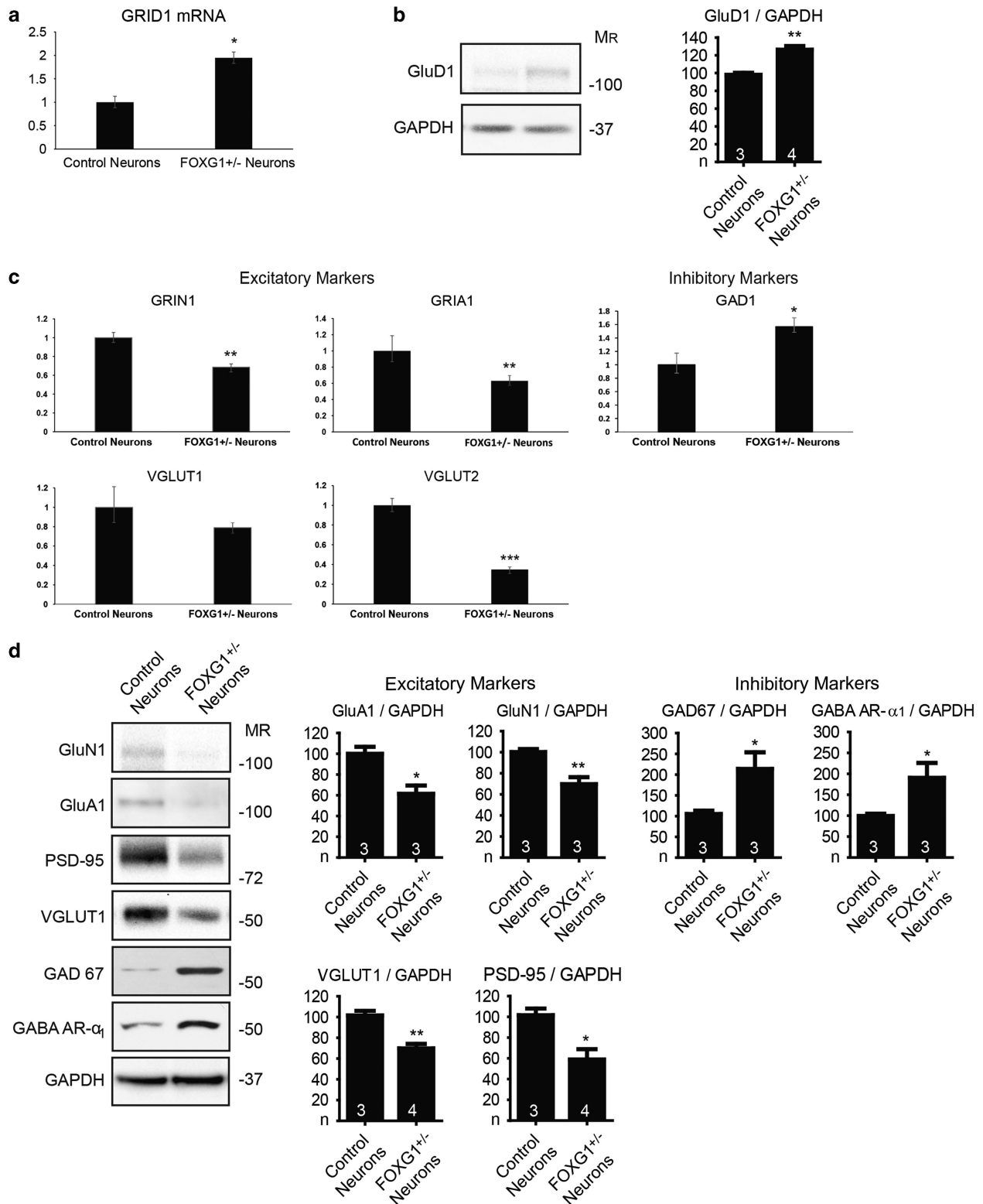
Considering the involvement of GluD1 in the development of excitatory and inhibitory synapses, we decided to evaluate the expression of a panel of inhibitory and excitatory synaptic markers through qRT-PCR and immunoblotting in terminally differentiated *FOXG1*<sup>+/-</sup> neurons.

We quantified mRNA levels for the glutamatergic markers *VGLUT1* and *VGLUT2*, which constitute the main two presynaptic vesicular glutamate transporters, for *GRIA1* encoding GluA1, one of the main two subunits of the AMPA-type glutamate receptor (AMPA), and for *GRIN1*, encoding GluN1, which is a central subunit of all NMDA-type glutamate receptors (NMDARs). The mRNA levels for all of these proteins showed a clear and statistically significant reduction; the only exception was *VGLUT1* that did not reach statistical significance ( $P = 0.09$ ), although it also exhibited a clear trend toward down-regulation (Figure 2c). At the same time, the mRNA levels of the inhibitory synaptic gene *GAD1*, encoding GAD67 that encodes the  $\gamma$ -aminobutyric acid (GABA)-synthesizing glutamate decarboxylase, showed a statistically significant increase of approximately twofold (Figure 2c,  $P < 0.05$ ).

To test whether these alterations in mRNA levels translate into changes in the protein levels we performed immunoblotting for most of these and a few other synaptic proteins. Once again, *FOXG1*<sup>+/-</sup> neurons showed a remarkable statistically significant increase (approximately twofold) in the two inhibitory synapse markers analyzed, the presynaptic GAD67 and the postsynaptic GABA<sub>A</sub> receptor subunit  $\alpha 1$  (Figure 2d). Inversely, the protein levels of presynaptic VGLUT1 and postsynaptic GluA1, GluN1 and PSD-95, which is the central postsynaptic structural protein of glutamatergic synapses, were significantly reduced (Figure 2d).



**Figure 1** Neuronal differentiation of iPSCs. (a) Overview of the neuronal differentiation protocol; the timing of critical steps is indicated. Specifically, the differentiation steps at which neuronal progenitors (PSA-NCAM+), differentiating (CD24+, d27/28 corresponding to day 15 of terminal differentiation) and mature neurons (CD24+, d42/43 corresponding to day 30 of terminal differentiation) were isolated for quantitative analyses are indicated. (b) Immunofluorescence analysis 24 h after dissociation of rosettes showed a large amount of dorsal telencephalic neural progenitors (NPCs day 12, expressing Nestin and SOX1). Neural differentiation of these cells mainly leads to the formation of glutamatergic excitatory neurons (VGLUT1 staining), with a small percentage of GABAergic inhibitory neurons (GAD65/67 staining).  $\beta_3$ -Tubulin (Tuj1) is used as a marker to stain all neurons in terminally differentiated cultures. DAPI was used to stain nuclei. Magnification:  $\times 20$ .



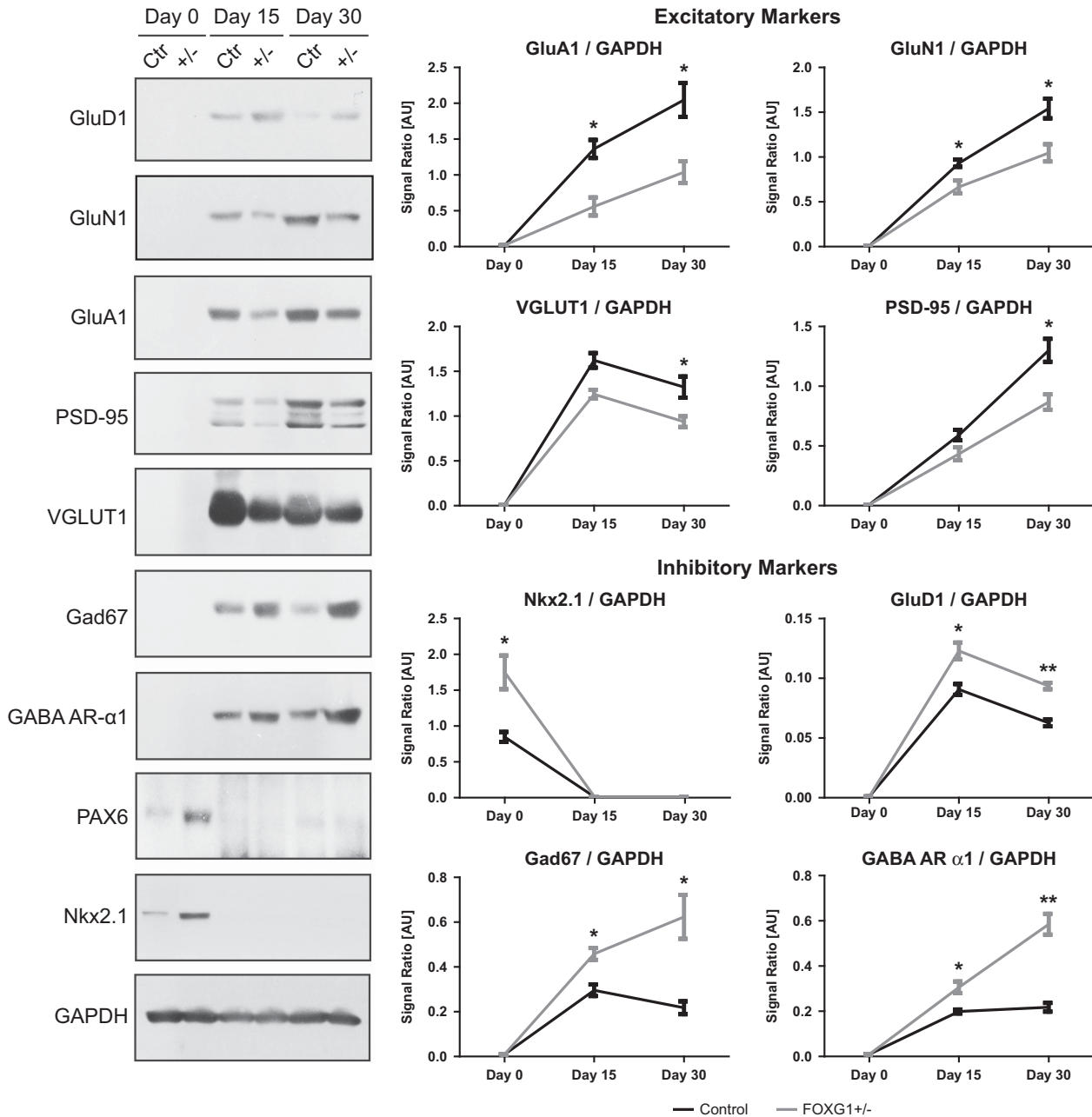
**Figure 2** Messenger RNA and protein levels of *GRID1* and key synaptic markers in control vs *FOXG1*<sup>+/-</sup> neurons. mRNA (a, c) and protein (b, d) levels of *GRID1* (a, b) and of selected excitatory and inhibitory markers (c, d) were evaluated. *GRIN1* encodes the NMDAR subunit GluN1, *GRIA1* the AMPAR subunit GluA1, *VGLUT1* and *VGLUT2* the two vesicular glutamate transporters and *GAD1* the GABA-synthesizing GAD67. To measure mRNA levels, real-time qRT-PCR was performed in three independent neuronal cultures obtained from one iPS clone per condition (one clone from each mutated patient and one control clone). Values in the y axis represent 2exp (-ddCt). Protein levels were determined by immunoblotting and quantified with Photoshop CS3 software (Adobe, San José, CA, USA); values on the y axis are the averages of signal ratio intensity expressed in arbitrary units. The number of different iPS clones analyzed is shown as n. Statistical significance was determined with Student's *t*-test (\**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.0005). The decrease in *VGLUT1* mRNA did not reach statistical significance but was substantial (*P*=0.09).



### Longitudinal analysis

It has been demonstrated that *FOXG1* plays an essential role in regulating timing and specification of the neuronal fate;<sup>10-13,41</sup> in particular, a progressive slowing of proliferation and precocious neurogenesis is observed in embryonic *Foxg1*<sup>-/-</sup> mouse brain whereas subtler and later alterations are observed in *Foxg1*<sup>+/-</sup> brain. As a consequence, we decided to investigate the possibility that the observed imbalance between excitatory and inhibitory markers resulted from a precocious onset of neuronal differentiation in mutated cells, resulting in an excitation/inhibition ratio typical of

more mature cultures. To this aim, we performed a longitudinal analysis by immunoblotting on lysates from neuronal progenitors, differentiating neurons (CD24+ at day 15 from the onset of terminal differentiation) and terminally differentiated cells to evaluate the evolution of marker expression along the differentiation process (Figure 3). Expression of the ventral progenitors marker NKX2.1 was higher in *FOXG1*<sup>+/-</sup> progenitors compared with control cells; its expression disappeared in differentiating neurons, irrespective of the genotype. All neuronal markers were absent at the progenitor stage and progressively increased from day 15 to day 30 of neuronal



**Figure 3** Longitudinal analysis of protein marker levels at different time points during neuronal differentiation. Protein levels of synaptic markers and transcription factors involved in neural differentiation were compared between control (black bars) and mutant cells (gray bars) across three different stages of *in vitro* neural differentiation: day 0 (neural progenitor cells), day 15 (intermediate differentiation) and day 30 (mature differentiated neurons). Each lane was loaded with equal amounts of proteins (50 μg). Statistical significance was determined using unpaired Student's *t*-test (\**P* < 0.05; \*\**P* < 0.01).

differentiation, with the exception of GluD1 and VGLUT1 that peaked at day 15; for GAD67, protein levels in control cells decreased from day 15 to day 30, whereas in *FOXG1*<sup>+/-</sup> cells the levels continue to grow. Comparison of control (black lane) and *FOXG1*<sup>+/-</sup> (gray lane) cells confirmed that overexpression of inhibitory markers and down-regulation of excitatory ones is stably maintained throughout the differentiation for all analyzed proteins.

#### Synaptic marker analysis in *Foxg1*<sup>+/-</sup> mouse brain

In order to strengthen a potentially causative relationship between *FOXG1* mutations and GluD1 upregulation, we decided to look at GluD1 expression levels in a *Foxg1*<sup>+/-</sup> mouse model. In order to mirror the developmental stage thought to be represented by the iPSC-derived neurons, we analyzed fetal mouse brains from developmental day E11.5. By comparing protein extracts from *Foxg1*<sup>+/-</sup> brains and litter-matched wild-type controls we observed a statistically significant increase in GluD1 levels (Figure 4).

We then quantified the various synaptic markers in the same set of samples. Remarkably, the protein levels of all synaptic markers followed a very similar pattern to what was observed in the iPSC-derived neuronal cells, with a significant increase in Gad67 and GABA<sub>A</sub> receptor  $\alpha 1$  levels and decrease in Vglut1, Psd-95 and GluA1 levels (Figure 4).

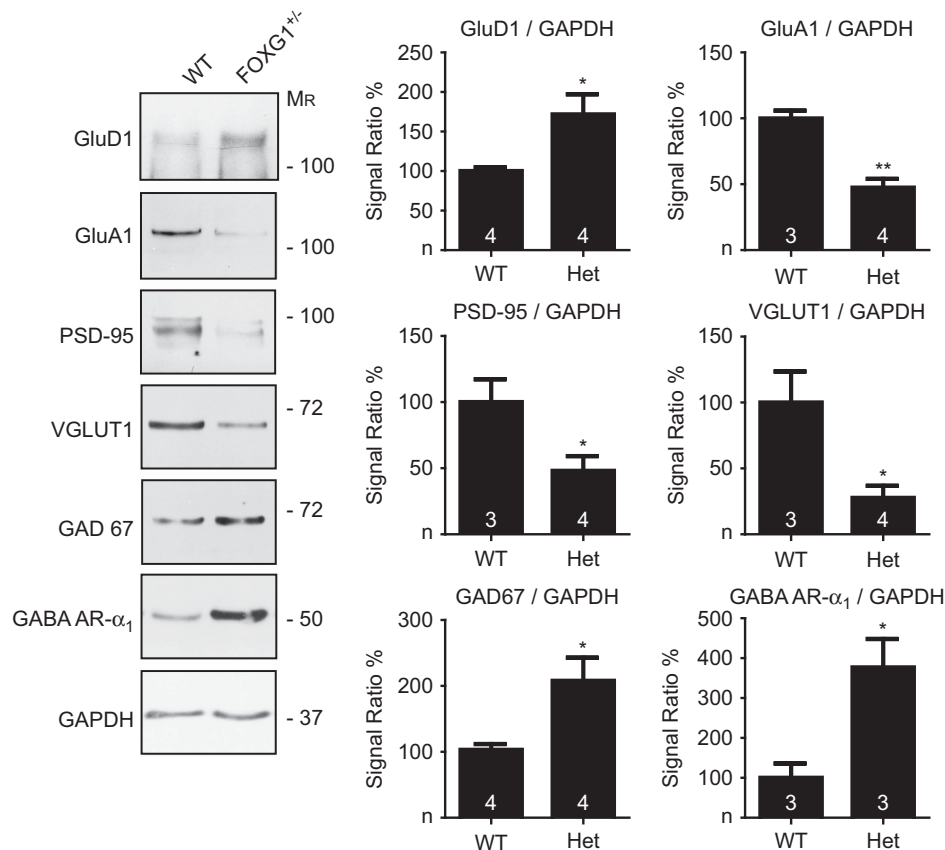
To know whether the changes in synaptic markers observed in *Foxg1*<sup>+/-</sup> fetal brains were only present during development or maintained into adulthood, we also analyzed adult brains (P70) from

*Foxg1*<sup>+/-</sup> mice. To our surprise, the levels of GluD1 and of all inhibitory and excitatory synaptic markers analyzed were significantly reduced in adult *Foxg1*<sup>+/-</sup> mice *versus* their litter-matched wild-type controls (Figure 5).

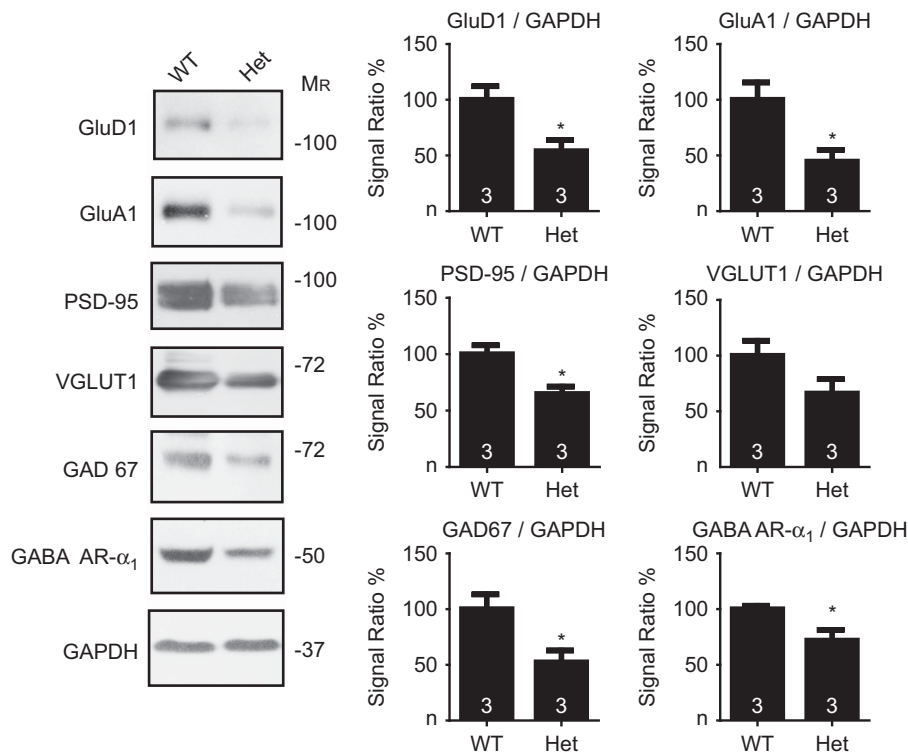
#### DISCUSSION

RTT is a severe neurodevelopmental disorder primarily due to mutations in *MECP2*. However, variants of this disease are also associated with mutations in either *CDKL5* or *FOXG1*. Patients with mutations in *FOXG1* are of particular interest because they lack the period of apparent normal development typical of classic RTT (therefore named the congenital variant).<sup>1,2</sup>

We generated for the first time neuronal cells from iPSCs obtained from individuals with *FOXG1* mutations and used them as an *in vitro* disease model to identify a potential disease mechanism in the congenital variant of RTT. Gene expression analysis in our *FOXG1*<sup>+/-</sup> iPSC-derived neurons showed an increase in *GRID1* mRNA levels as compared with controls (Figure 2a). This increase translated into an increase in GluD1 protein levels (Figure 2b). These data show for the first time that GluD1 is deregulated not only in classic RTT and the early-onset seizures RTT variant but also in the congenital variant due to *FOXG1* mutations, thus representing the first common alteration in cells with mutations in all genes associated with RTT spectrum disorders. Interestingly, genetic variation in the *GRID1* gene has been strongly associated with schizophrenia, supporting the idea that this



**Figure 4** Protein levels of synaptic markers in fetal *Foxg1*<sup>+/-</sup> mouse brains. Immunoblot analysis of inhibitory and excitatory synaptic markers in brain extracts from E11.5 mouse fetuses. Values on the y axis are the averages of signal ratio intensity expressed in arbitrary units. The number of mice used for each condition is reported as *n*. Statistical significance was determined with Student's *t*-test (\**P* < 0.05, \*\**P* < 0.01).



**Figure 5** Protein levels of synaptic markers in adult *Foxg1*<sup>+/-</sup> mouse brains. Analysis of a panel of inhibitory and excitatory synaptic markers in brain extracts from adult (P70) *Foxg1*<sup>+/-</sup> mice. Values on the y axis are the averages of signal ratio intensity expressed in arbitrary units. The number of mice used for each condition is reported as *n*. Statistical significance was determined with Student's *t*-test (\**P*<0.05). The decrease in VGLUT1 protein levels did not reach statistical significance but was substantial (*P*=0.0846).

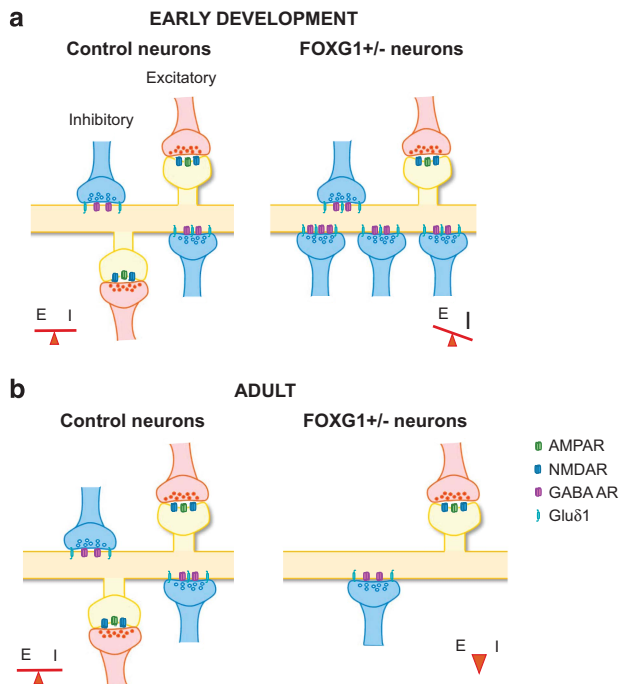
synaptic protein may be a general risk factor for neuropsychiatric disorders.

GluD1 is thought to be a synaptic adhesion molecule that can drive synaptic differentiation preferentially toward an inhibitory fate.<sup>28,29</sup> In addition, a recent study has identified GluD1 as a critical factor in the development and survival of inhibitory interneurons, as GluD1 KO mice have a profound loss of inhibitory cerebellar interneurons.<sup>30</sup> Therefore, we hypothesized that the increase in GluD1 levels in *FOXG1*<sup>+/-</sup> RTT neurons may correlate with an increase in the balance of inhibitory vs excitatory synapses. To test and gather evidence in support of our hypothesis, we selected two of the most representative inhibitory synaptic markers: presynaptic GAD67 (glutamate decarboxylase, coded by the gene *GAD1*) that synthesizes GABA, the main inhibitory neurotransmitter in the central nervous system, and the postsynaptic GABA<sub>A</sub> receptor  $\alpha_1$  subunit. Consistent changes in pre- and postsynaptic markers would instill confidence in the likely alteration of inhibitory transmission. Moreover, recent work has flagged *GAD1* as a gene of special interest for RTT as the transcriptional regulator MeCP2 directly binds to the *GAD1* promoter.<sup>42</sup> Our results show a profound upregulation of *GAD1* mRNA in differentiated *FOXG1*<sup>+/-</sup> neurons that was confirmed at the protein level in the same neurons (Figures 2c and d). In addition, GABA<sub>A</sub> receptor  $\alpha_1$  protein levels were significantly higher in mutant neurons as compared with the control lines (Figure 2d). Thus, both pre- and postsynaptic inhibitory markers show the same upregulation as GluD1, indicating that *FOXG1*<sup>+/-</sup> neurons have the propensity to form more inhibitory synapses (see model in Figure 6). At the same time, mRNA and protein levels were consistently reduced for several key excitatory synaptic markers, including VGLUT1, the AMPA-type glutamate receptor subunits GluA1, the NMDA-type receptor subunit

GluN1 and the excitatory post-synaptic scaffold protein PSD-95 (Figures 2c and d). These data support our hypothesis of a shift in the synaptic balance toward inhibition in *FOXG1*-mutant neurons (see model in Figure 6). Considering the correlation between the imbalance in synaptic markers and the upregulation of GluD1 levels in *FOXG1* mutant neurons and the function of GluD1 in promoting inhibitory synapses, it is tempting to speculate that GluD1 might play a direct role in causing this imbalance; however, at the moment, a direct link between the two events has yet to be established and further experiments will be necessary to determine whether a cause-consequence relation links GluD1 upregulation and the excess inhibitory synaptic markers observed in *FOXG1*<sup>+/-</sup> neurons.

The idea of an over-inhibition as a possible pathological mechanism in RTT is not completely new. In fact, it had been previously introduced by a study on *Mecp2*-mutant mice lacking epilepsy, where electrophysiological analysis of miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) revealed a reduction in excitatory but not in the inhibitory neuronal activity, resulting in an E/I imbalance in favor of inhibition.<sup>43</sup> In addition, a more recent study using human iPSC-derived RTT neurons with mutations in *CDKL5* described a reduction in excitatory synaptic markers that was recapitulated by knockdown of *Cdkl5* in rat primary hippocampal cultures. The same study showed how knockdown of *Cdkl5* in rat neurons also induced a reduction in the excitatory but not in the inhibitory neuronal activity, as measured by mEPSCs and mIPSCs, reinstating again the possibility of an over-inhibition in the pathogenesis of RTT.<sup>44</sup> In support of the over-inhibition hypothesis, two recent studies in mouse models already showed how chronic treatment with GABA<sub>A</sub> receptor antagonists at nonepileptic doses can rescue some behavioral phenotypes, such as





**Figure 6** Model of excitatory/inhibitory synaptic imbalances in *FOXG1*<sup>+/-</sup> neurons. Schematic model depicting possible scenarios of synaptic connectivity in *FOXG1*<sup>+/-</sup> neurons during embryonic brain development and in the adult brain. (a) Monoallelic mutations/deletions in *FOXG1* directly or indirectly cause an upregulation of GluD1 protein levels in both iPSC-derived neurons and fetal *Foxg1*<sup>+/-</sup> mouse brains that in turn might contribute to disrupt the synaptic balance toward inhibition. (b) In adult *Foxg1*<sup>+/-</sup> mouse brains both excitatory and inhibitory synapses could be reduced. In this model, the altered levels of inhibitory and excitatory synapses mirrors the alterations in the levels of pre- and postsynaptic markers experimentally observed.

motor coordination, episodic memory and synaptic plasticity, in both *Mecp2* duplication syndrome and Down syndrome.<sup>45,46</sup>

The common presence of seizures in the congenital variant of RTT may seem paradoxical to the hypothesis of increased inhibition. However, at least in the hippocampus of *Mecp2*-null mice it has been shown that a reduction in the basal excitatory tone decreases the brake on the rhythmic field potential activity of the neuronal networks that represents the outcome of both excitatory and inhibitory inputs. As a result, the overall hippocampal circuitry is more susceptible to excitatory stimuli, a condition that may be predisposing to seizures.<sup>47</sup>

Our recent work has shown how iPSC-derived neurons are more likely to model the early stages of neural development rather than the adult neural circuitry. In fact, comparing the transcriptome of human neurons derived from iPSCs with that of human neurons from postmortem samples at various stages of development, we found maximum correlation between the *in vitro* derived neurons and neurons of 10-week-old human fetuses.<sup>48,49</sup> To confirm our findings in an *in vivo* model, we thus decided to analyze the same set of synaptic markers in *Foxg1*<sup>+/-</sup> mouse brains from similar embryonic stages (E11.5).<sup>50,51</sup> The increase in GluD1 protein levels, initially determined in iPSC-derived *FOXG1*<sup>+/-</sup> neurons, was also found in the embryonic brains of *Foxg1*<sup>+/-</sup> mice (Figure 4). Moreover, the expression patterns of all the synaptic markers analyzed in the human-derived neuronal cells were mirrored in the E11.5 embryonic brains from *Foxg1*<sup>+/-</sup> mice (Figure 4). The high correspondence in synaptic

marker levels between the cellular and the animal model are the biochemical signature, confirming that iPSC-derived neurons best represent a specific stage of embryonic development (corresponding approximately to E11.5 in mice). The increase in GABAergic synaptic markers concurrent with the decrease in glutamatergic markers further supports our hypothesis of a shift toward inhibitory transmission in *FOXG1* mutants and more generally all known RTT mutations.

*FoxG1* is known to play an essential function in the early phases of telencephalon development; in *Foxg1*<sup>-/-</sup> mice, an abnormal progressive lengthening of cell cycle and precocious neurogenesis are observed as early as E10.5 and become evident at E11.5 in dorsal telencephalon, whereas no alteration is observed at earlier developmental phases (E9.5).<sup>13</sup> Considering that this is the stage of embryonic development that more closely correlates to our neurons, the observed excitation/inhibition imbalance could theoretically be the consequence of an alteration of the differentiation progression in mutated cells. In order to evaluate this possibility, we thus decided to also check marker expression in neuronal progenitors and in differentiating neurons at an intermediate time point (day 15) during the differentiation process. Our results indicate that the excitation/inhibition imbalance is consistently maintained during differentiation (Figure 3). Consequently, our observations suggest that the GABA/glutamate imbalance does not arise from an alteration in the timing of the differentiation process, but is a consequence of a shift of neuronal differentiation producing an excess of inhibitory neurons. However, more detailed analyses on cells at additional time points during the differentiation process will be necessary to confirm these results.

To test how the synaptic imbalance observed in fetal *Foxg1*<sup>+/-</sup> brains is affecting the synaptic balance in fully developed adult stages, we performed the same analysis of synaptic markers in adult (P70) *Foxg1*<sup>+/-</sup> mouse brains. In contrast to the developing brains, adult *Foxg1*<sup>+/-</sup> brains show a strong decrease in GluD1 and all the inhibitory and excitatory markers analyzed (Figure 5). This difference is presently difficult to explain. However, it is possible that the extensive neuronal apoptosis and synaptic pruning events that are known to occur in the brain during the perinatal period might result in an alteration of the previously established excitation/inhibition ratio, leading to the observed general decrease.<sup>52,53</sup>

In line with our model, a recent study showed that mice in which a loss of *MeCP2* was introduced only in a specific subset of forebrain GABAergic neurons recapitulate many of the phenotypes associated with RTT.<sup>54</sup> Interestingly, at an adult stage these animals showed a reduced inhibitory tone determined as a reduction in the expression of the *GAD1* and *GAD2* genes, as well as in GABA levels and inhibitory quantal size.<sup>54</sup> Other studies have also found a reduction in *GAD1* and *GAD2* levels in post-mortem tissue from schizophrenic and autistic patients.<sup>55,56</sup> To further complicate the situation, another study on *MeCP2*-deficient mice after analyzing both the GABA and glutamate neurotransmitter levels together with the mRNA and protein levels of key GABAergic and glutamatergic synaptic proteins showed how alterations of the GABAergic system may follow opposite directions depending on the age of the mice and on the brain region analyzed.<sup>57</sup> Similarly, opposite effects were also observed in the levels of excitatory synaptic markers in different brain areas of adult *Grid1* KO animals that accompanied a series of behavioral defects.<sup>58</sup> It is thus likely that disruptions in the excitatory/inhibitory balance during early phases of brain development may influence or even induce more complex and region-specific alterations of both excitatory and inhibitory circuits in the adult brain.

In conclusion, here we provided biochemical evidence that iPSC-derived neurons model a specific stage of embryonic brain

development (corresponding approximately to E11.5 in mice). By taking advantage of both a cellular and an animal model, we identified a synaptic imbalance toward inhibition as a possible new pathological event occurring in the brain of *FOXG1*<sup>+/-</sup> patients during embryonic development and its time-dependent shift toward a general decrease of brain synapses. Additional studies will need to clarify whether GluD1 levels are directly controlled by *FOXG1* and whether they directly regulate the excitatory/inhibitory balance, and this would open the pharmacological modulation of GluD1 or GABA<sub>A</sub> receptors as a possible strategy in RTT treatment.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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