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Epigenetic Control of the Notch and Eph Signaling Pathways by the Prion Protein: Implications for Prion Diseases

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Abstract

Among the ever-growing number of self-replicating proteins involved in neurodegenerative diseases, the prion protein PrP remains the most infamous for its central role in transmissible spongiform encephalopathies (TSEs). In these diseases, pathogenic prions propagate through a seeding mechanism, where normal PrP^{C} molecules are converted into abnormally folded scrapie isoforms termed PrP^{Sc}. Since its discovery over 30 years ago, much advance has contributed to define the host-encoded cellular prion protein PrP^{C} as a critical relay of prion-induced neuronal cell demise. A current consensual view is that the conversion of PrP^{C} into PrP^{Sc} in neuronal cells diverts the former from its normal function with subsequent molecular alterations affecting synaptic plasticity. Here, we report that prion infection is associated with reduced expression of key effectors of the Notch pathway in vitro and in vivo, recapitulating changes fostered by the absence of PrP^{C}. We further show that both prion infection and PrP^{C} depletion promote drastic alterations in the expression of a defined set of Eph receptors and their ephrin ligands, which represent important players in synaptic function. Our data indicate that defects in the Notch and Eph axes can be mitigated in response to histone deacetylase inhibition in PrP^{C}-depleted as well as prion-infected cells. We thus conclude that infectious prions cause a loss-of-function phenotype with respect to Notch and Eph signaling and that these alterations are sustained by epigenetic mechanisms.

Keywords Prion infection · Cellular prion protein · Notch · Eph · HDAC

Introduction

Prions are unique infectious pathogens composed of an abnormally folded protein known as PrP^{Sc}, standing for scrapie prion protein. PrP^{Sc} arises from the conversion of the host-encoded cellular prion protein PrP^{C}, itself a ubiquitous, glycosyl-phosphatidyl-inositol anchored (GPI) cell-surface molecule encoded by the *Prnp* gene [1]. While the self-

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templating replication of PrP^{Sc} is a well-established causative event at the root of transmissible spongiform encephalopathies (TSEs), our knowledge of the cellular and molecular mechanisms underlying prion-induced synaptic failure and ultimately neuronal loss is still far from complete (reviewed in [2]). Seminal observations [3–6] have altogether pointed to neuronal, GPI-anchored PrP^{C} as a mandatory relay of PrP^{Sc}-induced neurodegeneration, contributing to the current view that prions subvert the normal function of PrP^{C} to execute their toxicity. At the membrane level, PrP^{Sc}-PrP^{C} interactions may notably impact on the signal transduction pathways that are recruited upon binding of PrP^{C} to one of its various ligands (reviewed in [7, 8]). Depending on the effectors and/or pathway considered, the outcome may appear as a gain or loss of function. Grasping the accurate impact of PrP^{Sc} on PrP^{C} downstream effectors represents an arduous task in vivo in view of the multiplicity of cell types, partners, and signals at play. Notwithstanding, several over-activated signaling effectors have proven to be relevant targets to alleviate prion-induced neurodegeneration, such as NOX2 [9, 10], eIF2α [11], PDK1 [12], or ROCK [13]. In the later cases, it is noteworthy that PDK1 and ROCK over-activation are also observed in PrP^{C}-depleted cells [14, 15], highlighting common molecular changes in prion infection and PrP^{C} loss-of-function paradigms.

Assuming that prion-infected and prion-depleted cells may share more commonalities, we built upon our recent identification of Notch pathway alterations in absence of PrP^{C} [16] to evaluate the status of Notch effectors under prion infection. This pathway is composed of a set of transmembrane ligands and receptors whose interaction on adjacent cells promotes the proteolytic cleavage of Notch receptors and the nuclear translocation of their intracellular domain to regulated gene transcription [17]. Beyond its overriding role in stem cell self-renewal [17], it is now acknowledged that Notch signaling also plays an important role in mature neurons [18]. Our study takes advantage of the 1C11 neural cell line and its prion-infected derivatives, Fk-1C11 cells that chronically replicate the GSS-derived Fukuoka prion strain [19]. It also combines analyses on prion-infected mice. Beyond Notch alterations, we report convergent impact of prion infection and PrP depletion—in vitro and in vivo—on the expression of Eph receptors. Ephrin signaling, which relies on a panel of 14 Eph receptors that constitutes the largest family of receptor tyrosine kinases, and 8 ephrin ligands [20], plays major roles in both synaptic plasticity and neuronal activity [21]. We further provide evidence that inhibition of histone deacetylases (HDACs) sustains the recovery of Notch and Eph effectors in PrP-depleted and prion-infected cells. We conclude that prion infection and PrP^{C} loss of function share detrimental consequences on Notch and Eph signaling via epigenetic silencing.

Materials and Methods

Ethics Approval

Animal experiments were carried out in strict accordance with the recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 2010/63/EU) and all efforts were made to minimize suffering. Experiments were approved by the Local Ethics Committee on the Ethics of Animal Experiments of INRA of Jouy-en-Josas (permit numbers 12–034 and 15–056).

Animals

Tga20 mice were inoculated with uninfected 1C11 or infected Fk-1C11 cells as in [19], Fukuoka whole-brain homogenates (at the indicated dose) [22], or 139A prions [23]. Mice were euthanized when showing the first clinical signs. VPA (Sigma) was dissolved in 5% glucose and administered at 300 mg/kg through intraperitoneal injection, 6 days a week, from 50 days post-infection onward. Brains were dissected in ice-cold PBS and immediately frozen in liquid nitrogen for RNA and protein analysis. Analyses were carried out on n = 4 to 6 mice for each condition. E10.5 FVB/N (wild-type (WT)) and FVB/N $Prnp^{-/-}$ [24] mouse embryos from, respectively, WT × WT and $Prnp^{-/-}$ × $Prnp^{-/-}$ crossings were dissected in ice-cold PBS and immediately frozen in liquid nitrogen for RNA analysis.

Histoblot

Histoblot analysis was performed as in [25].

Reagents

All tissue culture reagents were from Invitrogen (Carlsbad, CA, USA). Recombinant rat Jagged1-Fc, IgG1-Fc, and EGF were from R&D systems (Minneapolis, MN, USA). TSA, VPA, U0126, and mouse monoclonal antibody against β-actin were from Sigma-Aldrich (St. Louis, MO, USA). FGF was from Eurobio (Courtaboeuf, France). 2M2P was from Thermo Fischer (Karlsruhe, Germany). Y27632 was from Tocris Bioscience (Ellisville, MO, USA). Anti-prion antibodies Sha31 and 12F10 were from SPI-Bio (Montigny le Bretonneux, France). Polyclonal rabbit antibodies against Jagged1 and Notch1 were from Cell Signaling Technology (Danvers, MA, USA).

Cell Culture and Treatments

1C11 cells and their PrP-null counterparts [15] and Fk-1C11 cells [19] were grown as previously described. Cells were

used at same passages. For cells grown on Jagged1, culture dishes were pre-coated with recombinant Jagged1-Fc at 3 μg/cm^2 or control IgG1-Fc in PBS. Y27632 and U0126 were used at 100 and 10 μM, respectively, for 24 h. We verified through western blot that at these concentrations Y27632 and U0126 efficiently reduce the levels of phosphorylated cofilin and phosphorylated ERK1/2, respectively. Appropriate TSA and VPA concentrations were adjusted through dose-response and kinetics analyses. Selected concentrations were 300 nM and 3 mM, respectively. At these concentrations, both TSA and VPA efficiently inhibit HDACs. 2M2P was used as negative control of VPA at the same concentration. Mouse neurospheres were obtained by whole brain dissection of E14 embryo from two lines from the same 129/ola strain: wild-type and *Prnp*$^{-/-}$ mice homozygous for a targeted null mutation in the *Prnp* gene [26]. Cells were grown as previously described in [27], i.e., in the presence of 20 ng/ml EGF and 20 ng/ml bFGF.

Preparation of Protein Extracts and Western Blot Analyses

Cells were washed in PBS and incubated for 30 min at 4 °C in NaDOC lysis buffer [50 mM Tris·HCl (pH 7.4)/150 mM NaCl/5 mM EDTA/0.5% Triton X-100/0.5% sodium deoxycholate] and a mixture of phosphatase (Thermo-Scientific, Waltham, MA, USA) and protease (Roche, Mannheim, Germany) inhibitors. Extracts were centrifuged at 14,000×*g* for 15 min. Protein concentrations in the supernatant were measured by using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Protein extracts (15 μg) were run on 12% Bis-Tris polyacrylamide gels (Bio-Rad, Marnes-la-Coquette, France), electrotransferred, and blotted onto nitrocellulose membranes. Immunoreactivity was visualized by enhanced chemiluminescence (Amersham Pharmacia Biosciences, GE Healthcare Europe, Velizy-Villacoublay, France). The protein levels were quantified with the ImageLab software, after acquisition of chemiluminescent signals with a Chemidoc digital imager (Bio-Rad, Marnes-la-Coquette, France). Signals were normalized to β-actin as a loading control for quantification.

PrP^{res} was extracted from 20% (*w*/*v*) tissue homogenates with the Bio-Rad TeSeE detection kit, and western blot analysis was carried out as in [28].

Isolation of Total RNA and Reverse Transcriptase-Polymerase Chain Reaction Analysis

RNA was isolated by using the RNeasy Extraction Kit (Qiagen, Limburg, Netherlands), as recommended by the manufacturer's instructions. For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, first-strand cDNA was synthesized with oligo(dT) primer and random 6mers, using the PrimeScript reverse transcriptase kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. Real-time PCR was performed using Absolute QPCR SYBR Green ROX Mix (Thermo-Scientific, Waltham, MA, USA) on a ABI PRISM 7900HT (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Real-time PCR analyses were performed with the SDS software 2.3 (Applied Biosystems). Primers used for the PCR reactions are shown in Supplemental Table 1.

Statistics

The results are reported as the means ± standard errors of the means (SEM). The unpaired Student's *t* test was used for comparisons. A p value < 0.05 was considered significant.

Results

Prion Infection Affects the Notch Pathway In Vitro and In Vivo

In order to evaluate the impact of prion infection on the Notch pathway, we first compared the expression of mRNAs encoding the Notch ligands Jagged1 (*Jag1*) and Jagged2 (*Jag2*); the Notch receptor *Notch1*, *Notch2*, and *Notch3*; and the Notch target genes *Hes1* in 1C11 infected cells versus their non-infected counterparts. To this purpose, we selected the Fk-1C11 #7 clone, hereafter referred to as Fk-1C11, which derives from 1C11 cells infected with the Fukuoka strain and accumulates moderate levels of PrP^{Sc} [19]. As shown in Fig. 1a, we found significantly reduced levels of both *Jag1* and *Jag2* mRNAs, as well as those encoding the three receptors *Notch1*, *Notch2*, and *Notch3* in Fk-1C11 undifferentiated cells as compared to the parental 1C11 cells. These changes were accompanied by a decrease in *Hes1* mRNA levels, suggesting that prion infection exerts a negative effect on Notch signaling in 1C11 cells.

In a next step, we sought to extend these in vitro observations to the in vivo situation. To keep as close as possible to our in vitro model, we exploited tga20 mice inoculated with prion-infected Fk-1C11 cells, as in [19]. Brains from Fk-1C11-inoculated mice were collected at the clinical stage (177 ± 10 dpi), and control mice inoculated with healthy 1C11 cells were euthanized at 180 dpi. Histoblot analysis (Fig. S1) confirmed the typical pattern of PrP^{res} deposits observed after tga20 infection with this strain [22].

Because potential region-specific changes in gene expression may be masked when analyzing the whole brain, we chose to study separately three regions: the brain stem, where the cell bodies of serotonergic neurons are located [29]; the cerebellum which is easily accessible and often affected in prion diseases; and the rest of the brain (referred to as "total brain"). To the opposite of our in vitro results, we found no

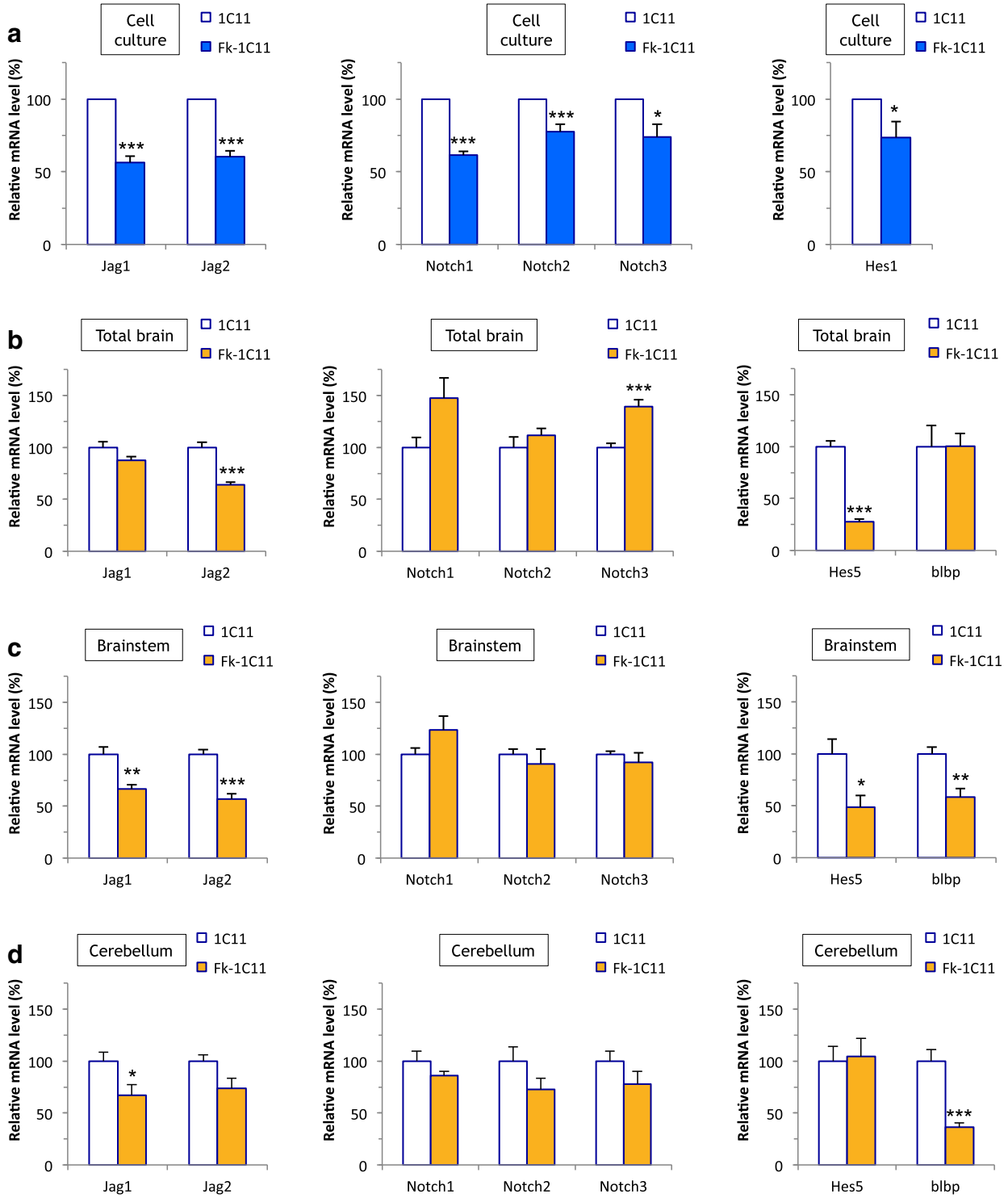


Fig. 1 Prion infection affects the Notch pathway in vitro and in vivo. Quantitative RT-PCR analysis of the expression of *Jag1*, *Jag2* (*left panel*), *Notch1*, *Notch2*, *Notch3* (*middle panel*), and the Notch target genes *Hes1* or *Hes5* and *Blbp* (*right panel*) was performed in prion infected Fk-1C11 cells versus control 1C11 cells (**a**) or in different brain regions from mice inoculated with Fk-1C11 cells or control cells: total brain (**b**), brain stem (**c**), and cerebellum (**d**). Results are expressed as means ± SEM of $n = 7$ cell preparations (**a**) or $n = 5$ mice (**b**–**d**). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control (Student's *t* test)

decrease in Notch receptor expression, and even a significant increase in *Notch3* mRNAs in the total brain (Fig. 1b, d, middle panels). However, whatever the region studied, we found a significant decrease of at least one of the Jagged ligands (Fig. 1b, d, left panels), as well as a decrease of at least one of the two Notch target genes *Hes5* and *Blbp* (brain lipid-

binding protein) (Fig. 1b, d, right panels). In the brain stem, we monitored concomitant reductions in *Jag1*, *Jag2*, *Hes5*, and *Blbp* (Fig. 1c, left and right panels). Thus, despite region-specific effects, which may relate to differential expression of Notch ligands and target genes according to brain structures (see Allen atlas of mouse brain; http://www.brain-map.org/), as well as to complex regulation according to the brain region considered, we consistently observed reduced expression of Notch pathway effectors. Finally, in line with the data obtained upon inoculation of Fk-1C11-infected cells, we monitored a trend towards reduced *Jag2* levels, together with increases in the Notch receptors *Notch1* and *Notch2*, and, most importantly, a 50% reduction in the expression of *Hes5* in the total brains of tga20 infected with 139A prions at clinical stage (52 ± 1 dpi) (Fig. S2a). Combined with the observations gained with Fk-1C11 cells, we may conclude that the Notch pathway is affected in response to prion infection.

Importantly, the defects in the Notch pathway monitored in prion-infected cells and mice recall those observed in stably PrP-depleted 1C11 cells as well as $Prnp^{-/-}$ embryos [16], supporting the view that PrP^{Sc} accumulation exerts a "loss-of-function" effect with respect to the Notch pathway.

Prions Affect the Eph/Ephrin Network In Vitro and In Vivo

Next, we sought to investigate potential Notch targets whose deregulation could participate to synaptic failure in prion pathology. We focused on Eph receptors and their ligands, since a subset are known transcriptional targets of Notch signaling in various contexts [30–33], including neural cells [34]. Our choice was also guided by previous reports showing under-expression of some Eph receptors in Alzheimer's disease, a disorder where PrP^{C} dysfunction contributes to neurodegeneration [35]. These include EphA4 and EphB2 [36, 37], whose restoration rescues cognitive functions in mice [38].

We thus assessed the impact of prion infection on the expression of Eph receptors and their ephrin ligands in Fk-1C11 cells (Fig. 2a). We first observed a drastic decrease (> 75%, $p < 0.001$) in the mRNA levels of a defined array of Eph receptors, namely, *EphA3*, *A4*, *A6*, *B2*, and *B6*, together with a more moderate but significant reduction of *EphA2*, *A8*, and *B3*, as compared with non-infected 1C11 cells. Fk-1C11 cells conversely exhibited increased levels of *EphA7* transcripts. *EphA5*, *A10*, and *B1* mRNAs were detectable neither in 1C11 nor in Fk-1C11 cells. Concerning the expression of ephrin ligands, it appeared less susceptible to prion infection, with only mild reductions in *efnA1*, *A2*, *A4*, and *B1* ($p < 0.01$).

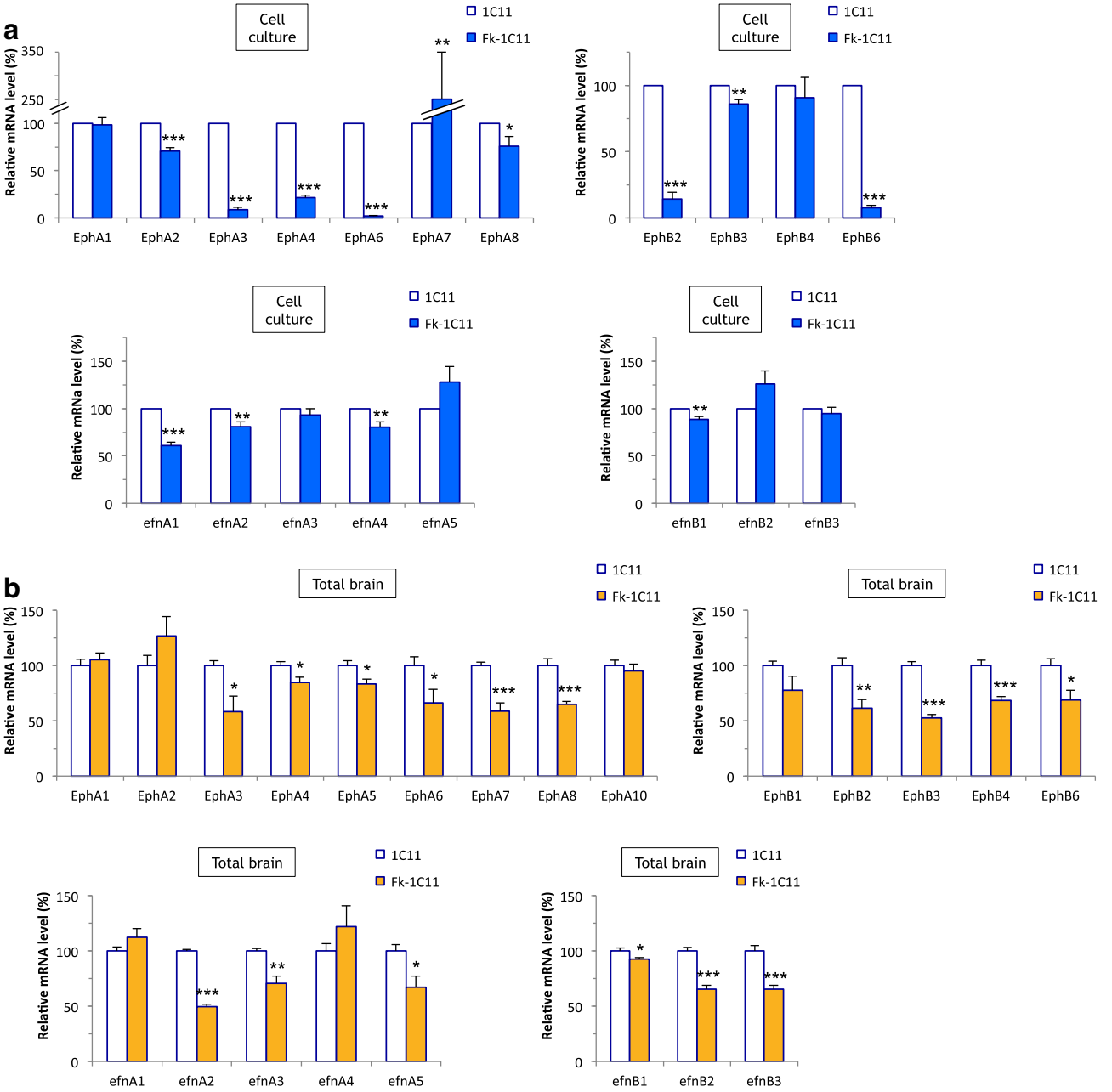
We went on to evaluate the outcome of prion infection in vivo on the ephrin pathway. We found significant reductions in the mRNA expression of 10 out of 14 Eph receptors as well as 6 out of 8 ephrin ligands in the total brain of mice inoculated with prion-infected Fk-1C11 cells versus control mice (Fig. 2b). Prion-induced changes were less marked in the brain stem, although the expression of ephrin ligands globally recapitulated that monitored in the total brain (Fig. 2c and Fig. S3a for detailed results). Regarding the cerebellum, six Eph receptors were significantly reduced, whereas only one ephrin ligand was decreased (Fig. 2c and Fig. S3b for detailed results). Of note, we may point out that there was a large overlap between the sets of Eph receptors with decreased expression in Fk-1C11 cells and in the total brains of mice inoculated with these prion-infected cells (Fig. 2c), with the exception of EphA7. Finally, most changes found in the total brains of Fk-1C11-inoculated mice (including reduced *EphA5*, *EphA6*, *EphA7*, *EphB3*, *EphB6*, *efnA3*, *efnB1*, and *efnB3* transcripts) were also recovered in the total brains of tga20 mice inoculated with 139A prions (Fig. S2b). Altogether, these results indicate that the Eph-ephrin axis is consistently affected in various models of prion infection, in line with transcriptomic data obtained in cerebellar organotypic slices [39] or wild-type CD1 mice [40] infected with RML prions.

PrP Depletion In Vitro Affects the Eph/Ephrin Pathway

In view of our initial hypothesis that impaired Notch signaling would contribute to reduced Eph/ephrin expression, we sought to analyze the status of these effectors under PrP depletion since we recently showed that it affects the Notch pathway [16]. In PrP^{KD}-1C11 cells that stably express a shRNA targeting *Prnp* mRNAs [15], we found that a panel of five Eph receptors encoding transcripts were barely detectable (> 98% reduction for *EphA3*, *A6*, *A7*, *B2*, and *B6*) while *EphB4* mRNAs were reduced to 11% of control (Fig. 3a). Some other milder changes were also noted among Eph and their ephrin ligands. We further confirmed that the absence of PrP^{C} impacts the Eph/ephrin axis in two other models of PrP^{C} depletion, namely, neurospheres (NSC) derived from $Prnp^{-/-}$ versus WT embryos (129/Ola genetic background) [27] (Fig. S4a), and dissected neural tubes from $Prnp^{-/-}$ versus WT embryos at embryonic day (E) 10.5 (FVB/N genetic background) (Fig. S4b). Thus, we consistently observed changes in the expression of transcripts encoding Eph receptors and ephrin ligands in three different models of PrP^{C} depletion (see the Venn diagram and the heatmap in Fig. S4c).

Importantly, the data obtained with PrP^{KD}-1C11 cells further allowed us to define a signature of five reduced Eph that was common to PrP^{C} loss of expression and prion infection both in vitro in the 1C11 cell system and in vivo in mice inoculated with Fk-1C11 cells (see the Venn diagram; Fig. 3b), four of which are severely decreased in vitro (> 85% reduction for *EphA3*, *A6*, *B2*, *B6* in both Fk-1C11 and PrP^{KD}-1C11 cells). Interestingly, nearly all changes monitored in PrP^{KD}-1C11 cells were recovered in either cells or mice infected with Fk prions (Fig. 3b, c).

In order to highlight our hypothesis regarding a Notch-Eph axis, we chose to implement the Notch data (from Fig. 1 and


a
Cell culture
1C11
Fk-1C11
Relative mRNA level (%)
EphA1
EphA2
EphA3
EphA4
EphA6
EphA7
EphA8
EphB2
EphB3
EphB4
EphB6
Relative mRNa level (%)
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efnA2
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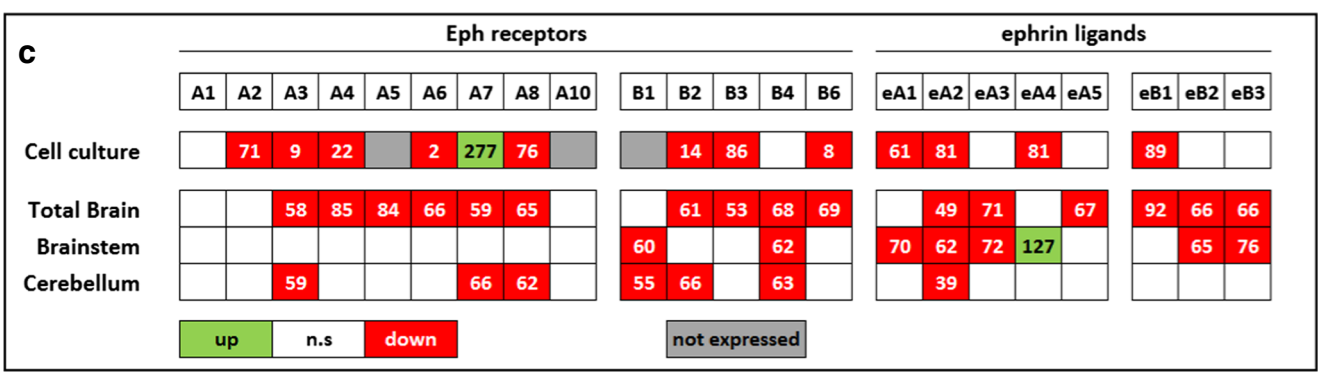
EphB1


c
Eph receptors
ephrin ligands
A1 A2 A3 A4 A5 A6 A7 A8 A10
B1 B2 B3 B4 B6
eA1 eA2 eA3 eA4 eA5
eB1 eB2 eB3
Cell culture
71 9 22 2 277 76
14 86 8
61 81 81 89
Total Brain
58 85 84 66 59 65
61 53 68 69
49 71 67 92 66 66
Brainstem
60 62 70 62 72 127 65 76
Cerebellum
59 66 62 55 66 63 39
up
n.s
down
not expressed

◀ **Fig. 2** Prion infection affects the Eph/ephrin network in vitro and in vivo. Quantitative RT-PCR analysis of the expression of genes of the EphA receptor family (*top left panel*), EphB receptor family (*top right panel*), ephrinA ligand family (*bottom left panel*), and ephrinB ligand family (*bottom right panels*) was performed in prion infected Fk-1C11 cells versus control 1C11 cells (**a**) or in total brain from mice inoculated with Fk-1C11 cells or control cells (**b**). Results are expressed as means ± SEM of $n = 5$ cell preparations (**a**) or $n = 5$ mice (**b**). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus control (Student's t test). **c** Heatmap summary of the changes in Eph and ephrin expression upon infection with Fk prions in vitro (Fk-1C11 cells) and in vivo (brain, brain stem, cerebellum), pointing to a common Eph network signature

[16]) into the Venn diagram and the heatmap summarizing Eph/ephrin expression in prion-depleted and prion-infected settings (Fig. S5a, b). These clearly illustrate the concomitant reduction of Notch and Ephrin pathways in these two conditions, in line with our working model.

Notch Activation Fails to Restore Eph Expression in PrP-Depleted Cells

Having shown a co-reduction of Notch and Eph pathway effectors in both prion-depleted and prion-infected contexts, we chose to directly test our hypothesis that Eph receptors may be transcriptional targets of Notch. To this purpose, we exposed PrP^{KD}-1C11 cells to recombinant Jagged1 since we recently reported that a 24-h culture on Jagged1-coated dishes strongly induces the expression of Notch pathway effectors in PrP^{KD}-1C11 cells through a positive feedback loop [16] (see also Fig. 4a, left panel). We focused on the expressions of *EphA3*, *A6*, *B2*, and *B6* transcripts, since these four receptors are severely reduced in Fk-infected and PrP^{KD}-1C11 cells (see Figs. 2a and 3a, respectively). Contrary to our expectations, in PrP^{KD}-1C11 cells, the restoration of the Notch pathway failed to boost the expressions of *EphA3*, *A6*, *B2*, or *B6* receptors (Fig. 4a). When performing the same analysis with 1C11 cells, we however observed increased levels of *EphA3* and *B6* mRNAs (Fig. S6). As for *EphB2* expression, it was insensitive to Jagged1, while *EphA6* transcripts were significantly decreased (Fig. S6). More generally, out of the 19 Eph/ephrin components expressed in 1C11 cells, 4 were transcriptionally induced in response to Jagged1, while 4 were repressed (Fig. S6). Thus, while Notch positively regulates some Eph/ephrin effectors in 1C11 control cells, the restoration of an active Notch axis in PrP-depleted cells is not sufficient to induce the transcription of these genes, suggesting that there may be other pathways exerting a negative control on their expression.

Inhibition of Signaling Pathways Over-Activated in Prion-Depleted and Infected Cells Is Not Sufficient to Restore Notch and Eph/Ephrin Pathways

Since our primary hypothesis was challenged by our results in Jagged-coated PrP^{KD}-1C11 cells, we asked whether there could be an alternative explanation for the concomitant reductions of Notch and Eph in prion-depleted and prion-infected contexts (see the Venn diagram and heatmap in Fig. S5a, b). In a first step, we sought to test whether these reductions could arise from a common signaling pathway that would be similarly deregulated in prion disease and under prion depletion. We investigated two signaling pathways that are over-activated in Fk-1C11 cells, namely, MAPK/ERK [41] and RhoA/ROCK [13], which lies upstream of the PI3K/PDK1/Akt pathway [12].

As already shown for ROCK [15], the MAPK-ERK pathway was found to be upregulated in PrP^{KD}-1C11 cells (data not shown). These observations prompted us to assess the impact of pharmacological inhibition of MEK (U0126) and ROCK (Y27632) on the Notch and Eph axes in PrP^{KD}-1C11 cells. However, neither U0126 nor Y27632 had a significant effect on the expression of the effectors that are the most affected by both PrP depletion and prion infection (Fig. 4b).

HDAC Inhibition Restores the Expression of Notch and Eph/Ephrin Effectors in Both PrP-KD- and Fk-Infected 1C11 Cells

Our failed attempt to restore Notch and Eph by inhibiting signaling pathways that are over-activated in PrP^{KD}-1C11 cells prompted us to study a possible involvement of histone deacetylases (HDACs) in the transcriptional repression of Eph and Notch effectors. The HDAC enzymes are epigenetic editors that can remove acetyl groups from lysine residues within the tail of histones, thus switching the chromatin from an active to a repressed state [42]. Noteworthy, once they have been recruited to a promoter/enhancer, HDACs can remain and stably maintain gene repression even if the transcriptional repressor that brought them in the first place is no longer present, as shown for the epigenetic silencing of *EphB3* by HDAC1 [43]. We might then postulate that in PrP^{KD}- or Fk-infected 1C11 cells, some initial events have eventually led to the stable repression of Eph and Notch genes by HDACs. Such an epigenetic silencing would notably accommodate our observation that *EphA3* and *EphB6* are insensitive to Jagged1 in PrP^{KD}-1C11 cells, whereas they are induced in Jagged1-coated 1C11 cells (Fig. 4a).

To challenge this idea, we first exposed PrP^{KD}-1C11 cells to trichostatin A (TSA)—a well-known HDAC inhibitor (HDACi), which, incidentally, has already been shown to upregulate both Notch [44] and Eph-ephrin [45] effectors. Of note, we observed a robust induction in the expression of *Jag2* in response to TSA (24 h) in PrP^{KD}-1C11 cells (Fig. 4c, left panel), which was accompanied by a raise in *EphA3* and *EphB2* mRNAs (Fig. 4c, right panel). Finally, *EphB6* transcripts were induced just above detection threshold under these conditions (Fig. 4c, right panel).

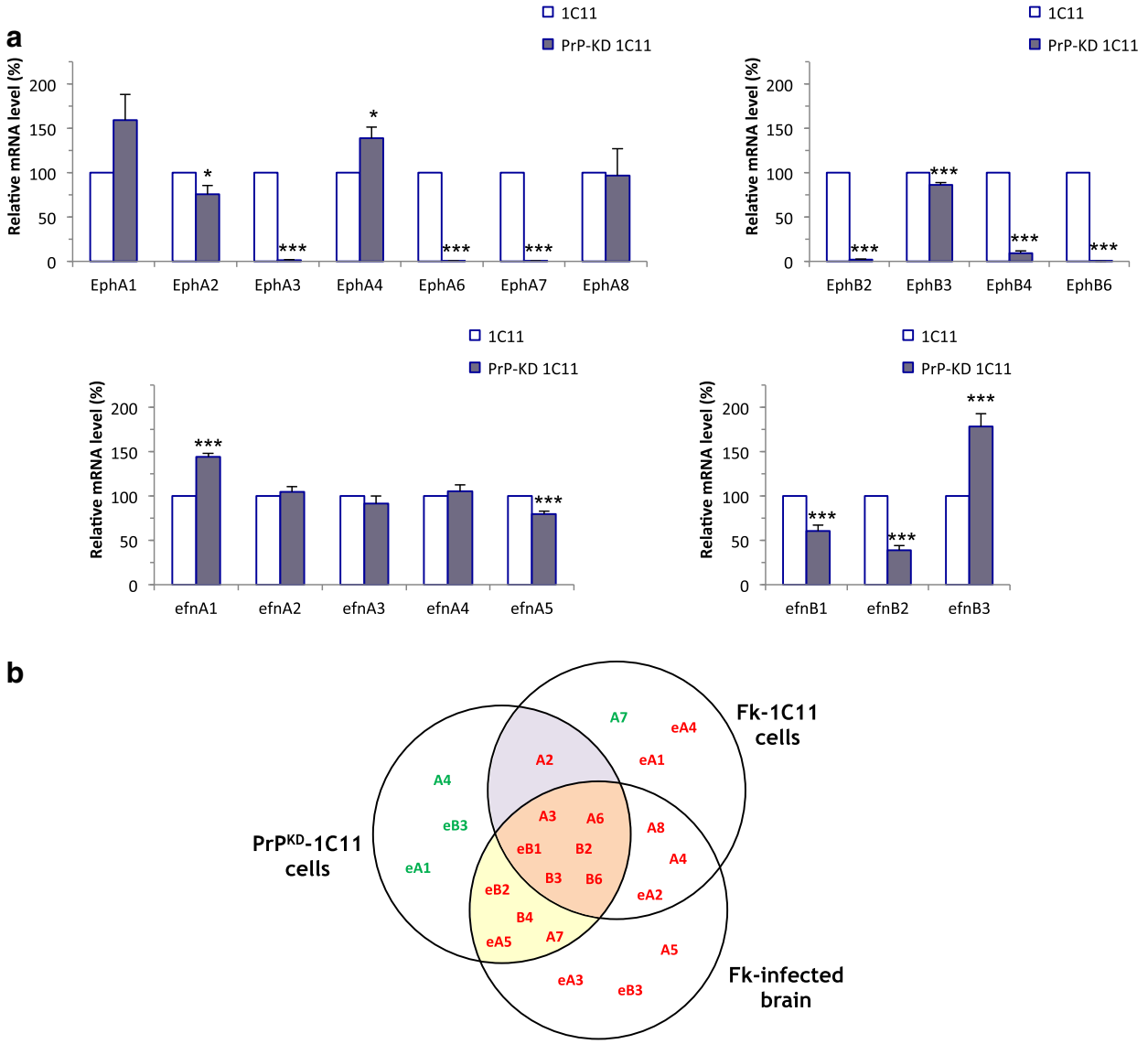


Fig. 3 The Eph/ephrin network is affected by PrP^C depletion in vitro. Quantitative RT-PCR analysis of the expression of genes of the EphA receptor family (*top left panel*), EphB receptor family (*top right panel*), ephrinA ligand family (*bottom left panel*), and ephrinB ligand family (*bottom right panel*) was performed in PrP^C-depleted PrP^{KD}-1C11 cells versus control 1C11 cells (**a**). Results are expressed as means ± SEM of $n = 6$ cell preparations. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control (Student's *t* test). Venn diagram (**b**) and heatmap (**c**) summary of the changes in Eph and ephrin expression upon PrP^C depletion in vitro (PrP^{KD}-1C11 cells) as compared to infection with Fk prions in vitro (Fk-1C11 cells) and in vivo (Fk-infected brain), highlighting common impacts of prion depletion and prion infection on the Eph network

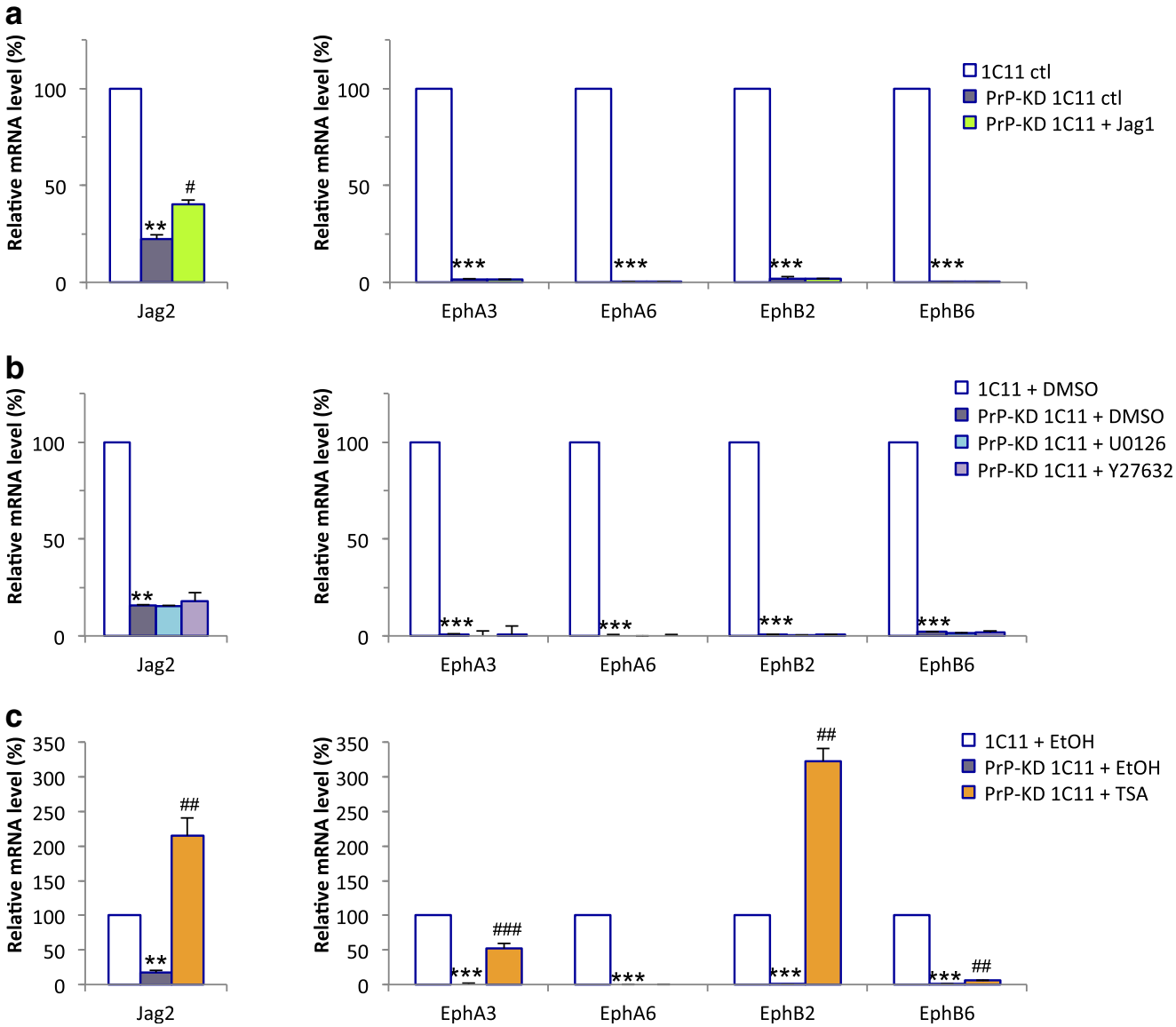


Fig. 4 Pilot analysis of various treatments on the expression of Jag2 and an Eph subset in PrP^{C}-depleted cells. **a** Quantitative RT-PCR analysis of the expression of genes encoding *Jag2* (*left panel*) and *EphA3*, *EphA6*, *EphB2*, and *EphB6* (*right panel*) in PrP^{C}-depleted PrP^{KD}-1C11 exposed to recombinant Jagged1 versus control 1C11 cells. **b** Quantitative RT-PCR analysis of the expression of genes encoding *Jag2* (*left panel*) and *EphA3*, *EphA6*, *EphB2*, and *EphB6* (*right panel*) in PrP^{C}-depleted PrP^{KD}-1C11 exposed to the MAPK pathway inhibitor U0126 or the ROCK inhibitor Y27632 versus control 1C11 cells. **c** Quantitative RT-PCR analysis of the expression of genes encoding *Jag2* (*left panel*) and *EphA3*, *EphA6*, *EphB2*, and *EphB6* (*right panel*) in PrP^{C}-depleted PrP^{KD}-1C11 exposed to the broad HDAC inhibitor TSA versus control 1C11 cells. Results are expressed as means ± SEM of $n = 2$ to 4 independent duplicates of cell preparations. $^{**}p < 0.01$, $^{***}p < 0.001$ versus control 1C11-EtOH cells; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus PrP^{KD}-1C11-EtOH cells (Student's *t* test)

To confirm and extend these pilot findings, we turned to another HDACi with potential clinical use, namely, valproic acid (VPA), which has notably been shown to upregulate Notch1 mRNA in a phase II study for the treatment of neuroendocrine tumors [46]. Again, we exploited PrP^{KD}-1C11 cells to adjust the dose and time of treatment (see the "Materials and Methods" section). Exposure of PrP^{KD}-1C11 cells to 3 mM VPA for 24 h was found to robustly increase the levels of acetylated histone H3 (AcH3) (data not shown), without inducing any toxic effect, contrary to higher doses (≥ 4 mM). As anticipated, 2M2P, a structurally related VPA analogue without HDACi activity [47], had no effect on acetylated H3 levels and was later used as a negative control. In agreement with data obtained in response to TSA, PrP^{KD}-1C11 cells treated with VPA exhibited increased levels of most effectors of the Notch pathway, as compared to cells exposed to the inactive congener 2M2P (Fig. 5a). As for the Eph/ephrin network, while VPA appeared to have more moderate effects than TSA, it notwithstanding exerted a positive impact on *EphA7*, and, most notably, *EphB2* expression (Fig. 5b). We may note that exposure of

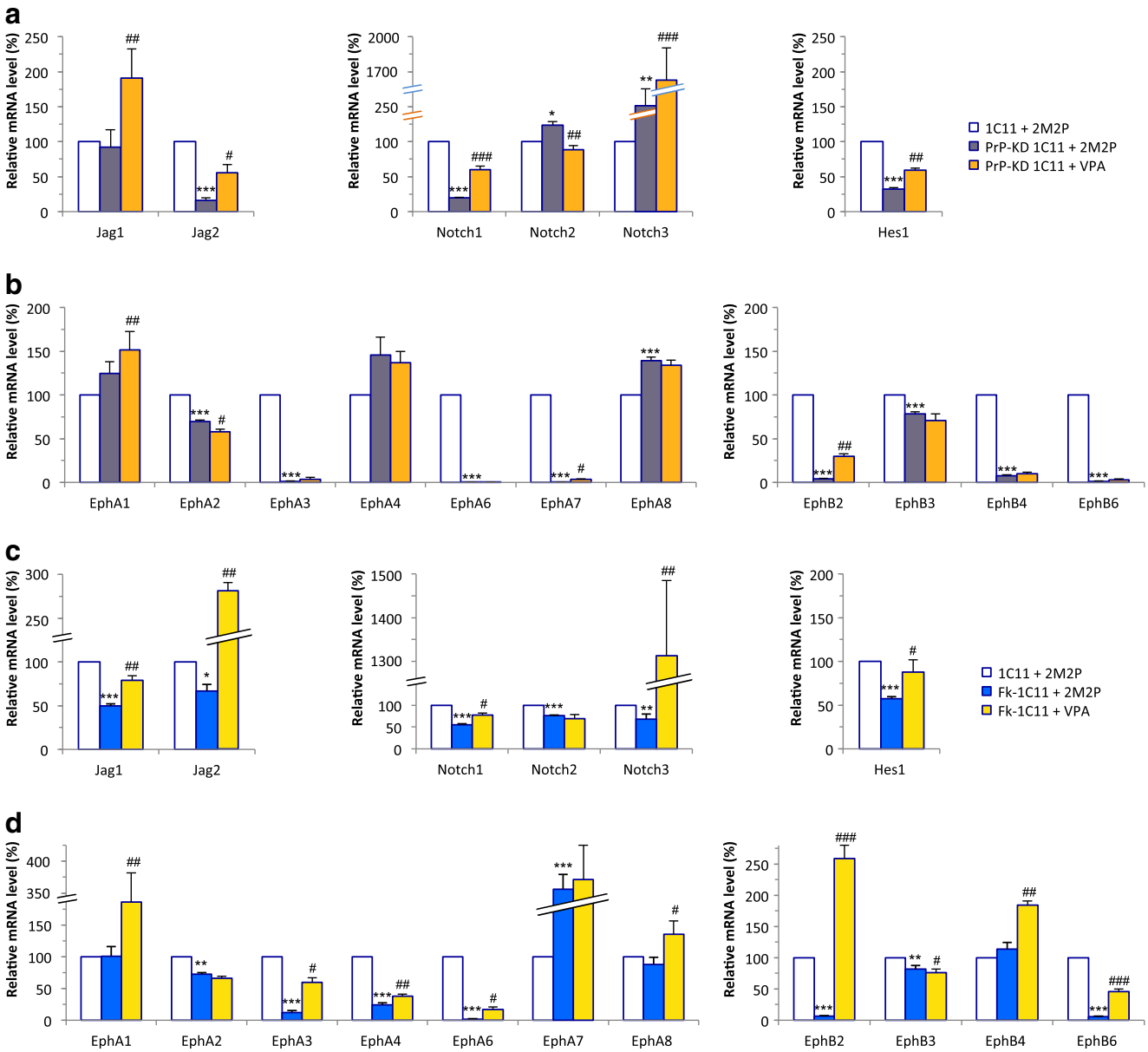


Fig. 5 Inhibition of HDAC exerts a positive impact on the Notch pathway and the Eph/ephrin network in both PrP^{C}-depleted and prion-infected 1C11 cells. **a**, **b** Quantitative RT-PCR analysis of the expression of genes encoding *Jag1*, *Jag2* (*left panel*), *Notch1*, *Notch2*, *Notch3* (*middle panel*), and the Notch target genes *Hes1* or *Hes5* and *Blbp* (*right panel*) (**a**) or the EphA receptor family (*left panel*), EphB receptor family (*right panel*) (**b**) in PrP^{C}-depleted PrP^{KD}-1C11 exposed to VPA versus control PrP^{KD}-1C11 and 1C11 cells exposed to 2M2P. **c**, **d** Quantitative RT-PCR analysis of the expression of genes encoding *Jag1*, *Jag2* (*left panel*), *Notch1*, *Notch2*, *Notch3* (*middle panel*), and the Notch target genes *Hes1* or *Hes5* and *Blbp* (*right panel*) (**c**) or the EphA receptor family (*left panel*), EphB receptor family (*right panel*) (**d**) in Fk-1C11 exposed to VPA versus control Fk-1C11 and 1C11 cells exposed to 2M2P. Results are expressed as means ± SEM of $n = 3$ cell preparations. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control 1C11-2M2P cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus PrP^{KD}-1C11-2M2P or Fk-1C11-2M2P cells (Student's *t* test)

PrP^{KD}-1C11 cells to VPA did not induce the expression of Eph transcripts that are not endogenously expressed in control 1C11 cells (e.g., *EphA10*), excluding the possibility of a non-specific impact of HDAC inhibition on global gene transcription.

We next applied the same treatment to prion-infected Fk-1C11 cells. The impact of VPA on the Notch pathway globally compared that observed with PrP^{KD}-1C11 cells, with increased levels of *Jag1*, *Jag2*, *Notch1*, *Notch3*, and the *Hes1* target gene (Fig. 5c). In agreement, we further found that the protein levels of Jagged1 and Notch1, both reduced in Fk-1C11 cells versus their non-infected counterparts—albeit not reaching significance for Jagged1—were significantly increased in response to VPA (Fig. S7). Finally, we successfully monitored upregulated levels of most Eph receptors under these conditions (Fig. 5d), including *EphA3*, *EphA6*, *EphB2*, and *EphB6*, which, according to our above results (see Fig.

3c), are severely reduced both under prion infection and prion depletion. This effect also applied to *EphA4* (Fig. 5d), itself not affected by the absence of PrP^{C}.

As a whole, these results disclose for the first time a link between the prion protein and epigenetic events.

Pilot Study to Assess the Effect of HDAC Inhibition in Prion-Infected Mice

We next carried out a preliminary study to probe the effect of VPA at late stage of prion pathogenesis in mice. Panels of tga20 mice were inoculated intra-cerebrally with Fukuoka whole brain homogenates at 0.01% and treated with 300 mg/kg VPA intra-peritoneally (ip) 6 days a week starting at 50 dpi (i.e., 3/4 of the incubation has elapsed). VPA is known to efficiently cross the blood brain barrier and was previously shown to exert a potent HDACi effect in the mouse brain after ip injection at this dose [48]. We may note that this prion infection model slightly differs from that used in our first experiments (inoculation with whole brain instead of cells, higher dose of inoculum), which could account for the different pattern of alterations monitored along the Notch and Eph pathways. Nevertheless, we consistently found defects in both pathways in the total brains (i.e., rest of the brain after removal of the brain stem and cerebellum) of mice infected with whole-brain-derived Fk prions (Fig. 6a, b), with significantly reduced expression of *Jag1*, *Jag2*, *Hes5*, and *Blbp* along the Notch pathway, and as well as decreased levels of mRNAs encoding *EphA3*, *EphA5*, *EphB1*, *EphB2*, *efna2*, and *efna5*. In non-infected controls, we found that VPA treatment had no impact on the molecular targets assessed. As for prion-infected mice, in contrast to our in vitro data, VPA treatment failed to upregulate the expression of genes of the Notch and Eph pathways (Fig. 6a, b). This lack of significant effect could account for the absence of impact of VPA on the onset of disease (Fig. 6c) or the levels of PrP^{Sc} (Fig. 6d). Notwithstanding, we observed a tendency towards an increase in the expression of several genes, including *Jag1*, *Hes5*, *Blbp*, and most notably *EphB2* ($p < 0.05$). These results suggest that a longer period of treatment and /or higher daily doses of VPA may allow a full restoration of the expression of those genes in prion-infected mice. Designing the proper conditions where VPA is effective at a molecular level is a prerequisite to assess whether defects in Notch and Eph pathways contribute to prion-associated neuropathogenesis.

Discussion

Over the past years, much evidence has accumulated supporting the notion that pathogenic prions divert PrP^{C} normal function. Based on this view, we here addressed one aspect of this subversion by focusing on the commonalities between prion infection and PrP^{C} loss of function. As an experimental approach, we first employed a cell-based strategy combining 1C11 cells, their prion-infected counterparts (Fk-1C11), and their PrP-depleted derivatives (PrP^{KD}-1C11). To assess the translational relevance of our in vitro findings, we secondly carried out analyses in brain material from mice inoculated with Fk-1C11 cells. Recently, this cellular model has proved valuable in bringing to light a common over-activation of the RhoA/ROCK/PDK1 pathway under both PrP^{C} depletion [14, 15] and prion infection [13]. In the present study, we show that PrP^{C}-depleted and prion-infected cells further share defects in Notch and Eph signaling and that these alterations can be mitigated upon inhibition of HDACs, highlighting an involvement of epigenetic mechanisms.

As observed in the absence of PrP^{C} in vitro and in vivo [16], we show that the impact of prion infection in cells and mice occurs at the level of transcripts encoding Notch effectors. These effectors include target genes (reduced expression of *Hes1* in vitro, *Hes5* and/or *Blbp* in vivo), providing strong evidence in view of a decreased Notch activity. This hypothesis is further supported by the significantly reduced protein levels of Notch1, together with a trend towards reduced Jagged1 levels, in Fk-1C11-infected versus control cells (Fig. S7).

In apparent contradiction with our results, two studies from the lab of S. Prusiner reported elevated levels of activated Notch (Notch intra-cellular domain (NICD)) in the context of prion infection (N2a cells and mice infected with RML prions) [49, 50]. In our experimental conditions, NICD levels were too low to yield conclusive data (unpublished observations), in line with NICD being detected in nuclear extracts in the study by Spilman et al. [50]. According to Ishikura et al. [49], the measure of total Notch1 levels mirrors that of NICD, which would argue for reduced NICD levels in the brains of Fk-1C11-infected mice. As a possible explanation for the difference between our results and those from the lab of S. Prusiner, the status of NICD under prion infection may exhibit subtle variations according to the region studied (neocortex in [49, 50] versus total brain, i.e., neocortex but also the olfactory bulb, corpus callosum, thalamus, hypothalamus, and hippocampus in our study). Besides, the detection of NICD may not unambiguously demonstrate canonical Notch pathway activation since the transcription of Notch target genes relies on the interaction of NICD with multiple partners including CBF1/RBP-J, as well as histone acetyl transferases [51]. An impact of prion infection and PrP^{C} depletion on one or several of those partners, hindering the formation of an active transcriptional complex, could therefore accommodate our results. Another plausible explanation that is worth considering would be the regulation of the expression of Notch effectors by signals beyond the Notch pathway itself. For instance, a positive regulation of several Notch target genes was reported in response to BMP4 in the context of oligodendrocyte differentiation [52]. Interestingly, the same study depicted that these effects are at least partly independent from

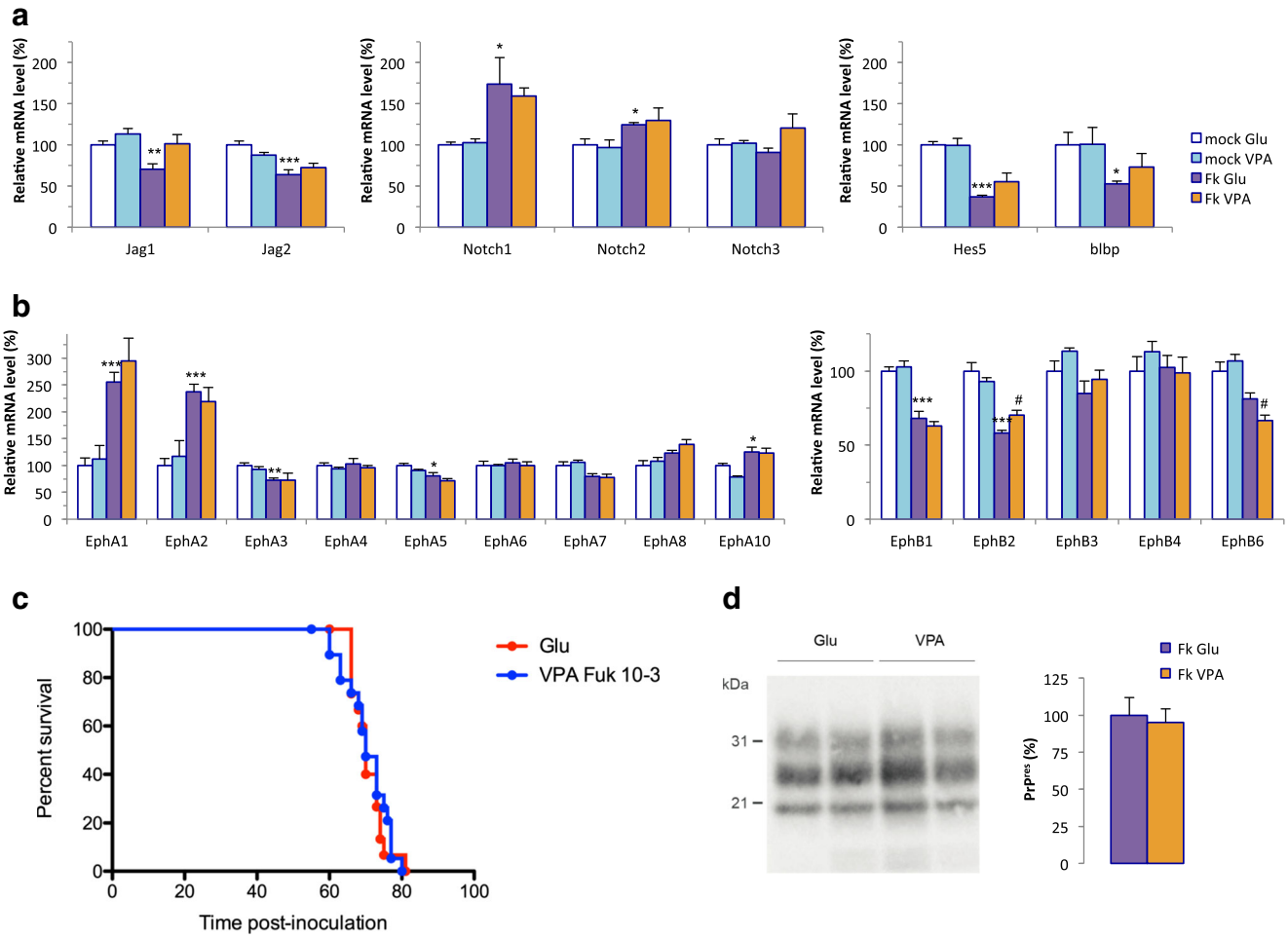


Fig. 6 Impact of VPA late treatment on prion pathogenesis in mice. **a**, **b** Quantitative RT-PCR analysis of the expression of genes encoding *Jag1*, *Jag2* (*left panel*), *Notch1*, *Notch2*, *Notch3* (*middle panel*), and the Notch target genes *Hes1* or *Hes5* and *Blbp* (*right panel*) (**a**) or the EphA receptor family (*left panel*), EphB receptor family (*right panel*) (**b**) in mice infected with Fukuoka whole-brain homogenates treated or not with VPA by intra-peritoneal injection starting 50 dpi and their respective non-infected controls. Results are expressed as means ± SEM of $n = 4$ to 6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control uninfected untreated mice; $^{\#}p < 0.05$ versus untreated prion-infected mice (Student's *t* test). **c** Survival curves of mice infected with Fukuoka whole-brain homogenates treated or not with VPA ($n > 15$ mice per group). **d** Representative Western blot for PK-resistant PrP^{Sc} in brain extracts of Fk-infected mice treated or not with VPA

Notch activation, while they involve a reduction of the binding of HDACs at the promoters of these genes. For these reasons, we believe that our results are not incompatible with the reported increased in NICD levels in prion-infected models. We may also note that, while different prion strains may have distinct outcomes on the Notch system, we monitored a 50% reduction in the levels of *Hes5* mRNA in the brain of mice infected with 139A prions (see Fig. S2a), fully consistent with the data obtained with Fk prions. Defects in the Notch axis under prion infection may contribute to synaptic dysfunction, since this pathway was reported to contribute to synaptic plasticity in hippocampal networks [53]. It may also account for the altered cell fate decision of adult neural stem cells in prion-infected mice, thereby impairing the endogenous brain repair machinery [54].

A second axis that we show here to be drastically impacted by the absence of PrP^{C} and prion infection is the Ephrin-Eph network. Similarly to Notch effectors, changes were monitored at the mRNA level. In this case, we analyzed the full repertoire of Eph receptors and ephrin ligands and recorded very drastic (> 85%) reductions in the expression of a defined set of Eph receptors (*EphA3*, *EphA6*, *EphB2*, and *EphB6*) under both PrP^{C} depletion and prion infection in the 1C11 cell system. Of note, our results obtained in neurospheres derived from *Prnp*$^{-/-}$ mice embryos (Ola/129 strain) as well as in dissected early neural tubes from *Prnp*$^{-/-}$ mice embryos (FVB/N strain) confirm that the Eph-ephrin pathway is strongly affected by the absence of PrP^{C} (Fig. S4a, b). With respect to prion infection using the Fk-1C11 model, our in vivo data corroborate our in vitro findings, as observed for the Notch pathway. They further substantiate a wider impact of prion infection in vivo on the Eph and ephrin axis, with reductions in 10 out 14 Eph as well as 6 out of 8 ephrins in total brain extracts. A likely explanation for the more extensive changes in vivo is the global contribution of

multiple cell types, compared to a defined, homogenous cell population in vitro. Although some of the effects may be strain specific, it is noteworthy that a large set of changes in the expression of Eph-ephrin effectors is recovered in the brains of mice infected with the 139A strain (Fig. S2b). In addition, *EphA3* features together with *EphA7* and *EphA8* among the genes that are downregulated 45 days after infection of cerebellar organotypic slices with RML prions [39]. In this respect, it is worth noting that we found the same Eph triad (*EphA3*, *EphA7*, and *EphA8*) to be decreased in cerebellar extracts of mice infected with Fk-1C11 cells (see Fig. S3b). Finally, alterations in the Eph-ephrin axis (decreased levels of *EphA3*, *EphA5*, *EphB2*, and *EfnB2* and increased levels of *EphB1*) were also found among the transcriptomic changes observed at clinical disease in wild-type CD1 mice infected with RML prions [40]. As a whole, these results support the view that deregulation of the Eph/Ephrin axis is a generic consequence of prion infection and that loss of PrP^C function may at least partly contribute to this phenomenon. In view of the prominent role exerted by these signaling molecules in synaptic plasticity [21], we speculate that the alterations in Eph signaling may take part to synaptic failure during prion pathogenesis. Among the targets of Eph-ephrin signaling, the stress-associated protein Fkbp5 has recently be shown to be transcriptionally upregulated in response to EphB2 reduced expression or cleavage [55, 56]. In line with this result, we found that mice inoculated with Fk-1C11-infected cells had a 4-fold increase in their brain *Fkbp5* mRNA level (not shown).

A third contribution of our study is the demonstration that HDAC inhibition alleviates the impact of PrP^C depletion and prion infection on Notch and Eph pathways. The reproducibility of the effects monitored in PrP^{KD}-1C11 with two unrelated drugs (namely, TSA and VPA), the use of VPA at a concentration relevant to HDAC inhibition [57], and the increase in AcH3 levels all confirm the specificity of the effects monitored, despite VPA targeting other effectors. A few studies have actually reported on the use of VPA in relation with prion infection, although the effects observed are disconnected from HDAC since the drug concentrations used were much below its HDACi IC50. The initial observation that VPA could increase the accumulation of PrP^{Sc} [58] was later refuted [59]. More recently, the report by Williams et al. that VPA protects from synaptic loss [60] is also based upon low doses (10 μM) of VPA and does not rely on HDAC inhibition since PIA, a non-HDACi structural variant of VPA, gives similar results. Instead, our experimental paradigm is close to that employed in [44] to induce expression of the Notch target gene Hes1 in a neuroblastoma cell line. Hence, HDACs appear to contribute to the negative regulation of Notch and Eph effectors in both PrP^C-depleted and prion-infected cells, raising the question as to how these two conditions may promote the epigenetic changes ultimately leading to gene silencing. We may suspect that under PrP^C depletion or prion infection, the disruption of PrP^C-dependent cascades leads to a downregulation of positive transcriptional activators, progressively shifting towards repression of enhancers. Such a mechanism, known as enhancer decommissioning, was recently described to cause *EphB2* and *EphB3* silencing in cancer cells [30, 61].

While delineating the mechanisms at play clearly requires deeper investigation, our study paves the way towards a detailed evaluation of the potential benefit of valproic acid in experimental TSEs. Our pilot study suggests that a longer treatment and/or higher dosage of VPA would be necessary to recapitulate in vivo the global increase in the expression of Notch and Eph pathway effectors that we obtained in vitro. Indeed, some studies have reported on the short-term effect of VPA in vivo, which may require multiple injections per day to avoid cycles of acetylation/deacetylation of histones in the brain [62, 63]. Another improvement may come along the development of encapsulated emulsions of VPA with better brain bioavailability [64]. Notwithstanding, it should be noted that, in the paradigm of our current study, VPA promoted a significant—although mild—upregulation of the expression of *EphB2*. This gene is of particular interest in the context of Alzheimer's disease (AD), in which the Aß amyloid peptide diverts PrP^C-dependent signaling to execute some of its toxic effects [35, 65]. Indeed, *EphB2* mRNA levels are decreased in the hippocampus of hAPP mice as well as AD patients and the restoration of EphB2 expression through viral vectors rescues cognitive functions in the hAPP mice [38]. Regarding therapeutic implications, it is of note that pharmacological targeting of HDACs features among the therapeutic strategies currently considered in the field of Alzheimer's disease (reviewed in [66]). Besides, VPA is a FDA-approved drug, which has been shown to upregulate Notch1 mRNA in a phase II study in cancer [46].

To conclude, our work discloses new prion-induced molecular alterations that are highly relevant to impaired synaptic function in TSEs. The proximity of changes induced by prion infection and PrP depletion provides a molecular framework to dissect the cascade(s) underlying defects in Notch and Eph axes. The positive outcome of HDAC inhibition in vitro warrants a deeper investigation of the therapeutic potential of epigenetic drugs to combat prion diseases.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interest.

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