Tubacin prevents neuronal migration defects and epileptic activity caused by rat Srpx2 silencing in utero

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Altered development of the human cerebral cortex can cause severe malformations with often intractable focal epileptic seizures and may participate in common pathologies, notably epilepsy. This raises important conceptual and therapeutic issues. Two missense mutations in the sushi repeat-containing protein SRPX2 had been previously identified in epileptic disorders with or without structural developmental alteration of the speech cortex. In the present study, we aimed to decipher the precise developmental role of SRPX2, to have a better knowledge on the consequences of its mutations, and to start addressing therapeutic issues through the design of an appropriate animal model. Using an in utero Srpx2 silencing approach, we show that SRPX2 influences neuronal migration in the developing rat cerebral cortex. Wild-type, but not the mutant human SRPX2 proteins, rescued the neuronal migration phenotype caused by Srpx2 silencing in utero, and increased alpha-tubulin acetylation. Following in utero Srpx2 silencing, spontaneous epileptiform activity was recorded post-natally. The neuronal migration defects...
and the post-natal epileptic consequences were prevented early in embryos by maternal administration of tubulin deacetylase inhibitor tubacin. Hence epileptiform manifestations of developmental origin could be prevented in utero, using a transient and drug-based therapeutic protocol.

Keywords: developmental epilepsy; Srpx2; neuronal migration; tubulin acetylation; in utero prevention

Introduction

Developmental malformations of the cerebral cortex with often intractable focal epileptic seizures represent a major medical and scientific issue (Guerrini et al., 2008; Barkovich et al., 2012). Early developmental cortical alterations may also participate in common pathologies with little or no structural neuroimaging anomaly, including autism, dyslexia, speech impairment and epilepsy; this raises important conceptual and therapeutic issues (Ben-Ari, 2008; Manent and LoTurco, 2012). Genetic studies have identified several molecular causes of abnormal cortical development (Rubenstein and Rakic, 1999; Guerrini et al., 2008). In particular, tubulins, microtubule-regulating proteins and alpha-tubulin acetylation modifiers play a crucial role in various cortical disorders (Keays et al., 2007; Creppe et al., 2009; de Nijs et al., 2009; Heng et al., 2009; Jaglin and Chelly, 2009; Jaglin et al., 2009; Kumar et al., 2010; Valiente and Marin, 2010; Wynshaw-Boris et al., 2010; Manzini and Walsh, 2011), including polymicrogyria, which is characterized by an excess of small gyri (Jansen and Andermann, 2005). Bilateral perisylvian polymicrogyria is a developmental malformation of the speech cortex. It is at the severe end of a continuum of epileptic syndromes where subtle and transient to severe and permanent speech and language impairments occur (Rudolf et al., 2009). At the benign end, Rolandic (Sylvian) epilepsy is the most common epilepsy syndrome in childhood. We had previously reported on two inherited missense mutations in the SRPX2 (sushi-repeat containing protein, X-linked 2) gene in two families with Rolandic epilepsy, with verbal dyspraxia (p.N327S mutation) or with bilateral perisylvian polymicrogyria (p.Y72S mutation), respectively (Roll et al., 2006). SRPX2 is a secreted protein expressed in the Rolandic area (Roll et al., 2006). Importantly, the direct causal role of p.N327S has been recently challenged by the co-inheritance, in most patients of the corresponding family, of another missense mutation in the glutamate receptor subunit gene GRIN2A (Lesca et al., in revision), raising an important issue about the respective participation and the interaction of those SRPX2 and GRIN2A mutations in the variable epileptic, speech and cognitive components of the phenotype.

SRPX2 might strongly interfere with the development and with the functioning of the cerebral cortex. Generally, proteins with sushi domains, such as SEZ6, GABA(B1a) receptor subunit, or LEV-9, can have important (patho)physiological roles in the CNS (Gunnersen et al., 2007; Gendrel et al., 2009, Biermann et al., 2010). Although the first molecular networks linked to SRPX2 have been identified recently (Royer-Zemmour et al., 2008; Roll et al., 2010; Bruneau and Szepetowski, 2011), there is a crucial need to decipher the precise developmental role of this protein and to have a better knowledge of the possible pathological mechanisms related with its mutations, in particular p.N327S. The use of rodent models, including in utero RNA silencing approaches, has been shown to be very efficient in deciphering major aspects of brain cortex development, and in understanding the pathophysiology of various brain disorders (Bai et al., 2003; Paracchini et al., 2006; Ramos et al., 2006; de Nijs et al., 2009; Rubenstein, 2011). In this study, we used an in utero RNA interference approach (Bai et al., 2003) to demonstrate that SRPX2 influences neuronal migration in the developing rat cerebral cortex. Analysis of the human wild-type and mutant SRPX2 proteins indicated that an impairment of neuronal migration and of alpha-tubulin acetylation might participate in disorders of the speech cortex in human. Maternal administration of the tubulin deacetylase inhibitor tubacin prevented the abnormal neuronal migratory phenotype and the post-natal neuronal epileptiform activity caused by Srpx2 silencing in utero.

Materials and methods

Constructs

Full-length wild-type and mutant human SRPX2 coding sequences were generated from previous constructs (Roll et al., 2006) using the hsSRPX2-F/hsSRPX2-R PCR primers (Supplementary Table 2). The full-length coding sequence of rat Srpx2 was amplified by reverse transcription PCR (mSrpx2-F/mSrpx2-R primers) (Supplementary Table 2) from adult rat brain total RNA. PCR fragments were inserted into pCAG-IRES-GFP (pCAGIG). For short hairpin Srpx2 silencing constructs, target sequences were selected in the rat Srpx2 gene (Genbank NM_001108243) (Supplementary Fig. 2). Triple-point-mutated short hairpin RNA (m3shSrpx2) counterparts were used as respective controls. All oligonucleotide-based inserts were subcloned into mU6pro (Yu et al., 2002). Commercially available sh-PLAUR silencing and corresponding control constructs were purchased (Qiagen). The Mec-17 overexpression construct (Li et al., 2012) has been reported previously. All constructs were verified by Sanger sequencing (GATC Biotech). All animal experimentations were conducted with respect to the European Communities Council Directives, and with licences from the French Department of Agriculture and the local Veterinary Authorities.

Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription PCR was carried out with the appropriate primers (Supplementary Table 2) in rat C6 cells transfected with pmaxGFP and with either short hairpin Srpx2 construct (5’shSrpx2 or 3’shSrpx2) or triple-point-mutated short hairpin RNA (5’m3shSrpx2 or...
3’ mShRNA vector (mU6pro), and analysed as described in Supplementary Fig. 2B and C.

Patients and Sanger sequencing

Thirty-six unrelated sporadic cases with various related disorders of the Rolandic epilepsy spectrum (atypical Rolandic epilepsy, Rolandic epilepsy with verbal dyspraxia, perisylvian polymicrogyria) were collected. Genomic DNAs were extracted from venous blood samples using a BAC33 kit (GE Healthcare). All experiments were conducted in accordance with the Declaration of Helsinki and all procedures were carried out with the adequate understanding and written informed consent that were obtained from the parents for their children and for themselves, according to the appropriate bioethics law and ethical committees (n° 05/78, CPP Strasbourg Alsace 1). The 10 coding exons and the intronic margins of ELP4 transcripts (Genbank accession number NM_019040) were amplified by PCR and sequenced (GATC Biotech). Sequences were analysed using the Genalys 3.0 software. Primer sequences and reaction conditions are available upon request.

In utero electroporation

In utero injections and electroporations were performed as previously described (Carabalona et al., 2012) in embryos from timed pregnant rats (embryonic Day 15) that were anaesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg). Wistar rats (Janvier) were raised and mated at INMED Post Genomic Platform (PPGI) animal facility. For silencing and overexpression experiments, the appropriate constructs were co-elec-troporated with pCAGGS-mRFP (red fluorescent protein) at a ratio 3:1 (1.5 μg/0.5 μg). For rescue experiments, the silencing construct was co-electroporated together with the appropriate expression construct and the RFP expression vector (3:3:1 ratio). pCX-EGFP-N1 was used for electrophysiological analyses and for experiments on organotypic slices.

In situ hybridization

In situ hybridization was performed on cryosections of embryonic and post-natal rat brain tissues as previously reported (Pereira et al., 2008) and as further described (Supplementary Fig. 1).

Cell cultures, proteins and immunocytochemistry

Cell lines were grown and transfected according to standard procedures (Supplementary Figs 2, 6 and 7). Stably transfected Chinese hamster ovary (CHO) and U87MG cell lines were established and maintained as reported (Bruneau et al., 1997) (Fig. 4 and Supplementary Fig. 6). Protein production and secretion were obtained and estimated as described in Supplementary Fig. 6. Primary neuronal cells were prepared as reported (Roll et al., 2006) from cortices of 16-day-old rat embryos (E16) electroporated at embryonic Day 15 with the appropriate constructs (Supplementary Fig. 3). The tubulin acetylation analyses were performed with the appropriate primary antibodies (Supplementary Table 3), by western blotting (anti-mouse or anti-rabbit PA-conjugated secondary antibodies) after cell lysate protein extraction, and by immunocytochemistry (secondary antibodies conjugated to Alexa Fluor® 488, 555 and 647). Expression levels were estimated by western blot scanning with Epson Perfection 3200 Photo scanner or with the Genesys software of the G:BOX gel imaging system (Syngene). Immunocytochemistry images were captured with a fluorescence microscope (Zeiss Axio Imager Z2) with ApoTome attachment.

Morphological analyses and immunohistochemistry

Morphological and immunohistochemistry experiments were carried out as described previously (Royer-Zemmour et al., 2008) on coronal slices (100 μm, vibratome, Microm; 14–20 μm, cryostat, Leica) with the appropriate primary (Supplementary Table 3) and secondary (Alexa Fluor® 488, 555 or 647-conjugated anti-rabbit, anti-mouse, anti-rat, anti-goat or anti-guinea pig IgGs) antibodies. Hoechst 33258 (1:2000, Sigma) was used for nuclei staining. BrdU (50 μg/kg of body weight; Sigma) was injected intraperitoneally to the pregnant mother. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed as recommended (Promega). Images were acquired by using a Zeiss Axio Imager Z2 microscope with structured illumination (ApoTome) equipped with Zeiss AxioCam MRm camera and processed using Axiovision software or with confocal laser microscope (Leica TCS SP2) equipped with a CoolSNAP camera.

Time-lapse experiments

Organotypic slices of embryonic Day 17 rat forebrains were prepared and cultured for 1 day in Neurobasal® medium (Life Technologies), starting with brains electroporated 48 h before (embryonic Day 15) in control (GFP) or silencing (GFP + 5’ shRNA) conditions, as described (Heng et al., 2008; Sessa et al., 2010). For time-lapse experiments, the dishes were mounted in a CO2 incubator chamber (5% CO2, 37°C) fitted onto an inverted confocal microscope (LSM510, Zeiss). Repetitive acquisitions were performed every 15 min for up to 6–7 h in latero-dorsal regions of the cortex where ~30–40 successive ‘z’ optical planes spanning 60–80 μM were acquired. z-stacks were combined in ImageJ. Average velocity of migrating cells was obtained using the ImageJ plugin Manual tracking (F. Cordelières, Institut Curie, France).

Pentylenetetrazol-induced seizures

Pentylenetetrazol (PTZ) experiments were performed and analysed as described previously (Velisek et al., 1992). GABAergic antagonist pentylenetetrazol was dissolved in 0.9% NaCl buffer and administered to post-natal Day 14 rats through two intraperitoneal injections (25 mg/kg of body weight) with a 10 min interval between each. Rats were then immediately observed and video-monitored for 30 min. The occurrence of generalized tonic-clonic seizures, corresponding to the more severe score (Class 5) according to a previously defined scale (Velisek et al., 1992), was observed (Supplementary Videos 3 and 4).

Electrophysiological recordings from brain slices

Multisite extracellular recordings of spontaneous activity were performed using multi-electrode arrays made up of 60 planar microelectrodes (TiN/SiN, 30 μm electrode diameter, 200 μm pitch) arranged over an 8 x 8 square grid (Multi Channel Systems). After x 1200 amplification (Multi Channel Systems MEA 1060), signals were sampled at 10 kHz using the Multi Channel Systems data acquisition card controlled by the Multi Channel Systems MCRack software for data monitoring, acquisition and storage. After recording, the raw
signals obtained from each experiment were processed off-line by using custom software tools specifically developed in MATLAB (The Mathworks). Recordings were performed at 30–32 °C. Slices were maintained in dishes and perfused with oxygenated artificial CSF of the following composition (in mM): 126 NaCl, 3.5 KCl, 1 MgCl₂, 2 CaCl₂, 10 D(+)-glucose, 1.20 NaH₂PO₄, 26 NaHCO₃ (5% CO₂ / 95% O₂). The spontaneous activity was monitored and recorded for 30–120 min, starting 15–20 min after setting the slice in the recording chamber to let the slice adapt to the new environment and reach a stable level of activity.

For patch clamp recordings, standard whole-cell recordings were performed in coronal neocortical rat brain slices at room temperature (20–22 °C) from the soma of GFP labelled and non-labelled neurons using an EPC-9 (HEKA Elektronik) amplifier and filtered at 3–10 kHz. Slices in the recording chamber were perfused with oxygenated artificial CSF of the following composition (in mM): 126 NaCl, 3.5 KCl, 1 MgCl₂, 2 CaCl₂, 10 D(+)-glucose, 1.20 NaH₂PO₄, 26 NaHCO₃ (5% CO₂ / 95% O₂). The intracellular solution contained (in mM): 130 K-glutamate, 10 Na-glucuronate, 4 NaCl, 4 MgATP, 4 phosphocreatine, 10 HEPES, and 0.3 GTP (pH 7.3 with KOH). Biocytin (final concentration 0.3–0.5%) was added to the pipette solution to label the neurons from which recordings were obtained. Neurons were visualized by using infrared differential interference contrast and fluorescence video microscopy. Spontaneous postsynaptic currents were recorded for 15 min at the reversal potentials for GABAergic (0 mV) and glutamatergic (+70 mV) currents. All recordings were made in normal artificial CSF (1 mM Mg²⁺) without any pro-epileptic pharmacological drug. Stored data were analysed using the Mini Analysis 6.0.3 (Synaptosoft Inc) and Origin (MicroCal) software. To minimize potential sampling bias, the pups from at least three deliveries for each condition were studied.

Ex vivo and in vivo experiments with tubacin

Ex vivo rescue experiments were adapted from previously published in vitro studies (Sudo and Baas, 2010), with slight modifications. Briefly, tubacin (ChemieTek) or niltubacin (Enzo Life Sciences) (2.5 μM each) was added to the Neurobasal® medium of organotypic slices taken from SrpX2-silenced embryos 1 day after embryonic Day 15 electroporation. Slices cultured for 2 days were fixed after 24 h of tubacin treatment and images were acquired (confocal microscopy, LSM510, Zeiss). For the in vivo experiments, intraperitoneal administrations 24 and 48 h after in utero electroporations of tubacin or of the vehicle (dimethyl sulphoxide, DMSO) to the pregnant rats were performed according to the aforementioned ex vivo rescue experiments, and to previously reported in vivo procedures (d’YdeWalle et al., 2011) (detailed protocol available upon request). To control the delivery into embryonic brains after maternal intraperitoneal injections, brain extraction of tubacin was carried out using a procedure adapted from similar extraction methods (Zhou and Gallo, 2010). The presence or absence of tubacin fragment ions into the brain extracts after discontinuation of maternal treatment with tubacin or with DMSO (the vehicle), respectively, was detected by multiple reaction monitoring mass spectrometry on a Fast LC-MS/MS platform combining UHPLC Nexera and the tandem MS model (LC-MS triple quadrupole 8030), as recommended (Shimadzu) (Supplementary Fig. 10).

Quantifications

For cell counting analyses, at least three adjacent brain sections were analysed from each sample by ApoTome or confocal microscopy and ×5 to ×40 magnified fields were acquired. The distribution of cells was quantified as described (Elías et al., 2007) (Fig. 2B). Fluorescent neurons were counted by a specific plugin of ImageJ (F. Cordeliers, Institut Curie, France). For all experiments, 500 to 3000 cells were counted in each brain. The in vivo orientation of radially migrating neurons was performed with ImageJ in 159–161 individual cells randomly chosen in three brains for each experimental condition. Morphometrical analyses and reconstruction of neurons were performed using the ImageJ plugin NeuronJ. To evaluate ex vivo rescue efficiencies, fluorescence intensities were quantified in each organotypic slice from the corresponding z-stack after automated subdivision into five equal regions from ventricle to the pial surface, by a specific ImageJ plugin (Fig. 5B).

Statistics

An overview on all statistical data is given in Supplementary Table 1. Data were expressed as means ± SEM unless otherwise stated. Briefly, non-parametric Kruskall-Wallis testing was used to detect heterogeneous distribution between groups wherever appropriate (cell counting analyses, quantitative reverse transcription PCR experiments, western blot analyses, neuronal migration speed rates, morphometrical dendritic values, burst parameters), and was followed either by non-parametric Mann-Whitney test (two-tailed) with the appropriate adjustment for multiple comparisons whenever needed (Bonferroni), or by Dunn’s post test for multiple comparisons. Mann-Whitney test, two-tailed, was also used in the analysis of quantitative reverse transcription experiments, cell proliferation parameters, neuronal speed rates, and rescue experiments in organotypic slices. The parametric unpaired t-test (two-tailed), with Bonferroni correction whenever needed, was used in pentylenetetrazol-induced seizures experiments, in the estimation of embryonic rescue after tubacin treatment, in the analysis of apical dendrite orientations, in the analysis of bursting cell numbers, and in the mass spectrometry detection of tubacin in embryonic brain extracts after maternal treatments.

Results

In utero rat SrpX2 silencing leads to altered position of projection neurons in the developing cerebral cortex

In agreement with previous reverse transcription PCR experiments (Royer-Zemmour et al., 2008), expression of the rat SrpX2 gene was revealed by in situ hybridization in the developing telencephalon and in the post-natal brain (Supplementary Fig. 1A–C). Rat SrpX2 protein was also detected in the same stages by immunoblotting and by immunohistochemistry (Fig. 1A and B). SrpX2 showed expression at various embryonic stages from the proliferative ventricular/subventricular zones to the cortical plate and was notably detected in neural and neuronal progenitor cells (Fig. 1C–F and Supplementary Fig. 1D). SrpX2 expression was also shown at embryonic Day 20 (E20) in neuronal cells (Fig. 1F and G), notably along radial glial processes (Fig. 1H).
Cortical projection neurons are generated from the dorsal telencephalon in the ventricular/subventricular zones and migrate radially towards the pial surface through the intermediate zone to reach the cortical plate; the future neocortex. In order to investigate the role of Srpx2 in the development of the cerebral cortex, embryonic Day 15 rat embryos were electroporated in utero with control or with two specific short hairpin RNA constructs targeting the 5' (5'shSrpx2) and the 3' (3'shSrpx2)
untranslated regions of Srpx2, respectively (Supplementary Figs 2 and 3). Srpx2 silencing caused impaired positioning of electroporated projection neurons at embryonic Day 20 in the somatosensory cortex (Fig. 2 and Supplementary Fig. 4). The phenotypes of Srpx2 silencing were significantly different from the regular positioning of projection neurons obtained in control conditions (Fig. 2A and B and Supplementary Table 1). As the specific short hairpin RNAs yielded identical phenotypes, one of the two (5’shSrpx2) was used for all next experiments. At embryonic Day 20 ectopic Srpx2-silenced cells showed expression of molecular markers of either immature (Tuj1) or mature (NeuN) neurons (Fig. 2C and D). The phenotype caused by Srpx2 silencing persisted after birth (Fig. 2E). Specificity of silencing was confirmed by successful rescue electroporation experiments with expression of exogenous rat Srpx2 protein (Fig. 2A and B).

The phenotype caused by in utero Srpx2 silencing might be due to any perturbation in one of the complex sequential processes that are necessary for the cerebral cortex to develop properly, such as cell proliferation and cycle exit or cell migration (Marin and Rubenstein, 2003; Kriegstein, 2005). Consistent with the absence of any effect of SRPX2 on cell proliferation previously reported (Tanaka et al., 2009), examination of cell cycle markers BrdU (bromodeoxyuridine), Ki-67 and pHH-3 (phosphohistone H3) did not reveal any significant anomaly in Srpx2-silenced neural cell progenitors (Supplementary Fig. 5A–C). TUNEL assay and cleaved caspase 3 labelling did not reveal any influence on apoptosis (data not shown). No modification in the structure of the radial glia scaffold that drives the migration of projection neurons was observed (Supplementary Fig. 5D and E). Of note, various interneuron labellings did not show any obvious indirect effect on the trapping of interneurons in the ectopic area (data not shown). Although more subtle alterations in either of those mechanisms cannot be excluded, a direct role for SRPX2 in radial neuronal migration was privileged.

**Srpx2 silencing impairs neuronal migration**

Together with the role of SRPX2 in non-neuronal cell migration previously reported (Mlijkovic-Licina et al., 2009; Tanaka et al., 2009), this prompted us to investigate further the migration of postmitotic neurons ex vivo. Time-lapse imaging of cortical slices taken from rat embryos 2 days after 5’shSrpx2 electroporation at embryonic Day 15, confirmed the impaired migration of silenced neurons (Fig. 3A and Supplementary Videos 1 and 2). Morphological abnormalities consistent with a polarity defect were seen in Srpx2-silenced neurons. A subset of the cells did not extend a leading process appropriately, remained multipolar and were unable to migrate radially. Cells that were still able to adopt a bipolar morphology and to migrate towards the cortical plate, showed significantly reduced speed rates (7.42 ± 0.81 μm/h, n = 25 cells) as compared with control electroporated neurons (12.93 ± 1.26 μm/h, n = 25 cells) (P = 0.0002, Mann Whitney test, two-tailed), which, in turn, reached the previously reported speed rates of control bipolar neurons (13.7 ± 1.9 μm/h) (Crepe et al., 2009). At post-natal Day 7, altered orientation of the apical dendrite was observed (Fig. 3B). Also, reduced dendritic length and reduced number of dendritic branches were seen in Srpx2-silenced cortical neurons at post-natal Day 14 (see below).

**SRPX2 influences alpha-tubulin acetylation**

The morphological abnormalities and the neuronal migratory phenotype caused by Srpx2 silencing in utero were consistent with cytoskeletal defects. In particular, multiple neuronal migration pathways converge on alpha-tubulins (Kumar et al., 2010). The possible influence of SRPX2 on the acetylation of tubulin alpha was then tested in vitro. When CHO cells were cultured in a medium containing rat Srpx2 protein, a significant increase in the relative amount of acetylated alpha-tubulin was obtained as compared to the control condition without Srpx2 (Fig. 4A and B; Supplementary Fig. 6). Rat Srpx2 also led to increased acetylation of alpha-tubulin in other cell lines (Supplementary Fig. 7A). Similar results were obtained in the presence of the human SRPX2 protein (Fig. 4A–C; Supplementary Figs 6 and 7A). Notably, such an effect was not seen on the acetylation of the actin-interacting protein cortactin (Supplementary Fig. 7B), and SRPX2 did not lead to quantitative changes in the expression of either of the HDAC6 and SIRT2 deacetylases, or of the MEC17 (now known as ATAT1) and ELP3 acetyltransferases (Supplementary Fig. 7B). Srpx2 silencing in vitro led to cell-autonomous decreased expression of acetylated alpha-tubulin in AR4-2J cells (Supplementary Fig. 7C), and 5’shSrpx2 electroporation in utero also decreased the alpha-tubulin acetylation levels of the corresponding Srpx2-silenced brain areas (Fig. 4D) and of the derived primary neuronal cells (Supplementary Fig. 7D). We then asked whether the effects of SRPX2 could be influenced by its known receptor uPAR (plasminogen activator receptor, urokinase type) (Royer-Zemmour et al., 2008), which we detected in Srpx2-positive cells in the developing cortex (Supplementary Fig. 8A), and in primary neuronal cells derived from neural progenitors that had been electroporated in utero (Supplementary Fig. 8B). On the one hand, HEK293 cells do not express the SRPX2 receptor uPAR, as previously reported (D’Mello et al., 2009) and also verified here (data not shown); this indicated that SRPX2-driven tubulin-acetylation was not dependent on uPAR in these cells. Also, exogenous expression of uPAR at the cell surface of CHO cells did not significantly influence the increase in tubulin acetylation caused by SRPX2 (data not shown). On the other hand, silencing the endogenous uPAR-encoding PLAUR gene in stably-transfected U87MG cells led to a significant inhibition of the tubulin-acetylation effects of SRPX2 (Fig. 4E). Hence the signalling events linking SRPX2 to tubulin acetylation may involve either uPAR or other as-yet undetermined SRPX2 receptors in different cell types.

In contrast with their wild-type counterpart, no or only slight increase in alpha-tubulin acetylation was obtained with either of the two mutant SRPX2 proteins (Fig. 4A–C; Supplementary Fig. 6). The possible existence of a link between those mutations on the one hand, and the impaired radial neuronal migration on the other hand, was then further explored in vivo. Expression and rescue experiments were performed with the wild-type and
Figure 2  In utero Srpx2 silencing leads to altered positioning of projection neurons in the developing rat brain cortex. (A) Distribution of red fluorescent protein-positive cells in rat brain cortices (coronal sections) at embryonic Day 20 (E20), 5 days after electroporation of: pCAGGS-mRFP (RFP, red fluorescent protein) alone or with either of mU6pro control plasmid or specific (5’shSrpx2, 3’shSrpx2) or mutant ineffective (5’m3shSrpx2, 3’m3shSrpx2) Srpx2 short hairpin RNA constructs, and of: pCAGGS-mRFP with rat Rn.Srpx2-GFP expression construct either alone or with 5’shSrpx2 construct. Subregions were defined as in (B). (B) Significant changes in the distribution of cells at Tubacin prevents developmental epilepsy Brain 2013: 136; 2457–2473 (continued)
mutant human SRPX2 constructs. Expression of mutant p.Y725-SRPX2 had no effect on its own on the regular positioning of projection neurons at embryonic Day 20 (Supplementary Fig. 9A). Co-electroporation with 5'shSrpx2 showed that, in contrast with wild-type human SRPX2, p.Y725-SRPX2 had lost the ability to rescue the phenotype caused by Srpx2 silencing (Supplementary Fig. 9B). Altogether this indicated a loss-of-function mechanism. The other SRPX2 mutation was then tested. Single electroporation of p.N327S-SRPX2 construct caused by itself an altered and persistent pattern of neuronal positioning that was significantly attenuated by competitive overexpression of wild-type SRPX2 (Supplementary Fig. 9A–C), arguing for a dominant-negative effect, as predicted (Roll et al., 2006).

**Administration of tubacin during pregnancy prevents the post-natal epileptiform activity caused by Srpx2 silencing in utero**

Consistent with the influence of SRPX2 on alpha-tubulin acetylation, the phenotype caused by Srpx2 silencing was significantly rescued in vivo with vector-based overexpression of Mec-17 alpha-tubulin acetyltransferase (Akella et al., 2010; Li et al., 2012) (Fig. 5A). The impaired neuronal migration was also significantly rescued ex vivo when organotypic slices taken from Srpx2-silenced embryos were cultured in the presence of tubacin, a specific inhibitor of the alpha-tubulin deacetylating enzyme HDAC6 (Haggarty et al., 2003) (Fig. 5B). Niltubacin, the inactive homologue of tubacin, had no effect.

The possibility that pharmacological targeting of tubulin acetylation may also prevent in vivo the developmental phenotype caused by in utero Srpx2 silencing was then questioned. Beforehand, the ability to deliver tubacin into the brains of naive embryos after short intraperitoneal administration to their mother during pregnancy, was demonstrated by multiple reaction monitoring mass spectrometry after tubacin extraction (Supplementary Fig. 10A–C). Increased alpha-tubulin acetylation could be detected in most but not all tubacin-treated embryonic brains (Supplementary Fig. 10D); this was not unexpected, given the inherently variable and transient biochemical consequences of the therapeutic protocol used here. Tubacin was then injected at embryonic Days 16 and 17 to three pregnant rats having been electroporated on embryonic Day 15, Srpx2-silenced embryos (n = 19). The injections dramatically improved the neuronal migratory phenotype at embryonic Day 20 (Fig. 5C; Supplementary Table 1). Indeed, 15 of 19 Srpx2-silenced embryos treated with tubacin were fully or partially rescued, whereas the vehicle (dimethyl sulphoxide, DMSO) had no improving effect (0/12 embryos from three pregnant rats) (P = 0.000016, Fisher’s exact test, two-tailed) (Fig. 5D). Consistently, there were significantly less ectopic Srpx2-silenced neurons in post-natal Day 14 rats electroporated with 5'shSrpx2 and treated with tubacin in utero, as compared with the untreated silencing condition (Fig. 6A). The aforementioned morphological abnormalities seen in Srpx2-silenced cortical neurons at post-natal Day 14 were also significantly improved (Fig. 6B and C). Of note, no side-effect on the proliferation (data not shown) or on the post-natal positioning of other late-migrating neurons (Supplementary Fig. 10E) was detected. Overall tubacin given during pregnancy had long-term beneficial influence on the neuronal positioning and morphology of Srpx2-silenced neurons in the offsprings.

Developmental malformations of the cerebral cortex are frequently associated with epileptic activity (Guerrini et al., 2008; Andrade, 2009; Manent et al., 2009). Furthermore, both the SRPX2 mutations were found in the context of focal epileptic seizures in human (Roll et al., 2006). Higher risk to chemically-induced seizures of post-natal Day 14 rats subjected to 5'shSrpx2 in utero electroporations (Supplementary Fig. 11A and B; Supplementary Videos 3 and 4), suggested abnormal functioning of the networks comprising Srpx2-silenced neurons. Consistently, microelectrode array extracellular recordings in acute neocortical slices taken from post-natal Day 14 Srpx2-silenced rats, revealed spontaneous bursts, mostly diffused over the area of the GFP (green fluorescent protein)-labelled cells in layers IV–V, in the absence of any proconvulsive agent (Supplementary Fig. 11C–E). Slices from control electroporated rats did not show such a bursting activity (Supplementary Fig. 11F). After tubacin treatment in utero, the Srpx2-silenced rat brain slices showed greatly reduced spontaneous bursting activity (Supplementary Fig. 11G).

The cellular basis of the high susceptibility to epileptiform activity caused by Srpx2 silencing in utero was then studied in slices by whole-cell voltage clamp recordings of spontaneous excitatory and inhibitory currents from neurons of cortical layers IV–V, according to the microelectrode array extracellular recordings (Fig. 7A–C). In Srpx2-silenced cerebral cortices, there was a significant increase in
the number of cells showing prominent spontaneous epileptiform activity (interictal-like bursts associated with both glutamatergic and GABAergic inputs) \((P < 0.0001\) for both, Fisher’s exact test, two-tailed), as compared to the control electroporation condition (Fig. 7D). Of note, spontaneous bursting activity has already been described in control conditions in a minority of neocortical neuronal cells in similar post-natal stages (Yassin et al., 2010). The in utero expression of the human mutant p.N327S-SRPX2 protein that caused a persistent alteration in neuronal positioning on its own (Supplementary Fig. 9A–C), was also associated post-natally with a significant increase in spontaneous bursting activity, whereas its wild-type counterpart had no significant effect (Supplementary Fig. 12A–C). Interestingly, when recordings were made simultaneously from pairs of Srpx2-silenced GFP-labelled neurons and their non-electroporated neighbours, synchronized bursts between the silenced and the non-silenced cells were detected. The parameters of the bursts (maximal amplitude and charge transfer) significantly increased in silencing conditions (Fig. 7A, B, E, F, H and I; Supplementary Fig. 11H and I). Importantly, glutamatergic integral burst charge densities significantly increased (Fig. 7G) whereas the increase in GABAergic integral burst charge densities did not reach statistical significance (Fig. 7J).

The neuronal electrophysiological activity of the offspring caused by Srpx2 silencing in utero, showed significant improvement after prenatal administration of tubacin; indeed, the number of bursting neurons and the electrophysiological parameters of spontaneous activity were either fully (glutamatergic bursts) or partially (GABAergic bursts), similar to control values (Fig. 7C–F, H and I; Supplementary Fig. 11H and I). Also, tubacin treatment prevented the increase in excitatory glutamatergic integral burst charge densities seen in Srpx2-silenced condition (Fig. 7G). Therefore, maternal delivery of tubacin during pregnancy prevented the
Figure 4 Wild-type, but not the mutant SRPX2 proteins, increase alpha-tubulin acetylation. (A–C) Acetylation status of cells cultured in conditioned medium with either rat (Rn.) Srpx2 or human (Hs.) wild-type (wt) or mutant (p.Y72S, p.N327S) SRPX2 proteins (Supplementary Fig. 6). CTL = control; TSA = trichostatin A (0.5 μM). (A) Immunocytochemistry experiments. TSA (positive control), Rn.Srpx2 and wild-type Hs.SRPX2 increase acetylation of alpha-tubulin (green) in CHO-K1 cells compared with total alpha-tubulin (red). The mutant proteins had no effect. (B and C) Western blot experiments. Rn.Srpx2 and wild-type Hs.SRPX2 increase the ratio of acetylated versus total alpha-tubulin levels in CHO-K1 (B). Similar results were obtained in U87MG cells (C). Mutant human proteins had little or no effect. Western blots were normalized with GADPH. Control conditions were set to 100%. Values ± SEM represent the mean of at least six independent experiments. (D) Srpx2 silencing in utero decreases alpha-tubulin acetylation. Western blot experiments with pooled proteins extracted from punches of fluorescent areas in embryonic Day 17 Srpx2-silenced embryos (5'shSrpx2) and in control (RFP)-electroporated embryos (n = 4 to 6 embryos each). Control conditions were set to 100%. Histograms represent the values ± SEM of eight independent pools for each. (E) PLAUR silencing inhibits the increase in alpha-tubulin acetylation caused by SRPX2 in U87MG cells. Left: Expression of the uPAR protein encoded by the PLAUR gene, was barely detectable in U87MG cells stably transfected with sh-PLAUR silencing construct. Control silencing construct (sh-CTL) had no effect. Right: Wild-type Hs.SRPX2 increased the ratio of acetylated versus total alpha-tubulin levels in U87MG cells stably transfected with sh-CTL; this effect was lost in U87MG cells stably transfected with sh-PLAUR. Western blots were normalized with GAPDH. Control conditions were set to 100%. Values ± SEM represent the mean of eight independent experiments for each condition.
Figure 5. Rescue of the embryonic defects by tubacin administration during pregnancy. (A) Mec-17 acetyltransferase significantly rescued in vivo the altered neuronal distribution seen at embryonic Day 20 (E20) after Srpx2 silencing in utero. Values represent mean ± SEM. V = ventricle. Mann-Whitney test, two-tailed. (B) Rescue of migration defects in organotypic slices (E16 + 2 days in vitro) taken from Srpx2-silenced embryos in the presence of tubacin (2.5 μM), as compared with its inactive homologue (niltubacin) applied to each adjacent slice (14 and 12 slices from 10 embryos, respectively). Fluorescence intensities reflecting cell positions were converted into grey values and measured across each slice. Histograms represent the mean ± SEM of fluorescence intensities in five regions (R1–R5), from the ventricle to the pia (normalized as a percentage). Mann-Whitney test, two-tailed. (C) Maternal tubacin administration after in utero electroporation rescued the altered neuronal cell distribution seen at E20 in the population of Srpx2-silenced (5′shSrpx2 + RFP) embryos (n = 19) DMSO.
embryonic migration disorder and its associated long-term epileptiform consequences.

Discussion

In the present study, the role of sushi-repeat containing protein SRPX2 in the development of the rat cerebral cortex was shown by demonstrating that the acute inactivation of Srpx2 in the developing cortex led to altered radial neuronal migration. Despite some obvious limitations common to most rodent models, such as the differences between the gyric human and the smooth rat cortices (Lui et al., 2011), and the inherent human-specific features of the speech cortex, our data strongly support a similar role for the human SRPX2 counterpart. We had previously reported on the two missense SRPX2 mutations that were studied here, in two different, albeit related epileptic disorders of the speech cortex (Roll et al., 2006). As previously mentioned, the direct causal role of the p.N327S SRPX2 mutation in the family with Rolandic epilepsy and verbal dyspraxia was very recently challenged by the detection of a familial co-inheritance of another missense mutation in the GRIN2A gene (Lesca et al., in revision), which we found in most, but not all, patients who had also inherited the p.N327S SRPX2 mutation. How those two GRIN2A and SRPX2 mutations might independently or synergistically influence the three epileptic, speech and cognitive components in this family represents a complex issue. From this viewpoint, the data that were obtained here demonstrate that the expression of the mutant p.N327S-SRPX2 protein actually leads to impaired development of the cerebral cortex and to post-natal epileptiform activity, which is consistent with the actual influence of this mutation in at least a part of the corresponding human phenotype. Overall, the present analysis of the two SRPX2 mutations indicated that they both can have strong and convergent functional impacts. Hence neuronal migration defects might not only participate in bilateral perisylvian polymicrogyria, as expected, but might also influence the phenotype of Rolandoic epilepsy with verbal dyspraxia. Indeed, various neuroimaging anomalies including white matter anomalies, enlargement of lateral ventricles, biopercular polymicrogyria (Lundberg et al., 1999; Gelisse et al., 2003), and more recently increased cortical thickness and grey matter volume including areas associated with language (Pardoe et al., 2013), have been observed in patients with Rolandic epilepsy.

Srpx2 silencing in utero led to persistent mispositioning of projection neurons and to spontaneous epileptiform activity in Srpx2-silenced neuronal networks post-natally. A similar situation was seen with expression of the dominant-negative mutant p.N327S-SRPX2 protein. This is in line with the paradigmatic developmental origin of various brain diseases and with the so-called ‘neuroarcheology’ concept (Ben-Ari, 2008). Interestingly, synchronized bursts were recorded from pairs of electroporated and non-electroporated neurons. The situation may somehow recall the abnormal network activity recorded in misplaced neurons and their target areas in layers II–III in a rat model of cortical heterotopia (Ackman et al., 2009). Not exclusively and despite the lack of a detectable non-cell autonomous effect of Srpx2 silencing on tubulin acetylation in vitro and on neuronal migration in BrdU-based experiments in vivo, altered migration of a subset of non-electroporated neuronal cells might also occur, particularly in the context of a secreted protein. It is noteworthy that the cell-autonomous action of a secreted protein (BDNF, brain-derived neurotrophic factor) on neuronal cell polarity has already been demonstrated (Cheng et al., 2011). Nevertheless, increased excitatory glutamatergic bursting activity was observed in the Srpx2-silenced rat brain slices. Hence our findings are consistent with the increased susceptibility to chemically-induced seizures seen here. Our data also suggest that the present rat model of altered migration of projection neurons may at least partly recapitulate the pathophysiology of the seizures caused by early alteration of the cerebral cortex in human.

The dynamics of the microtubule cytoskeleton plays a crucial role in neuronal migration. We not only show here that SRPX2 influences neuronal migration, but also that it increases acetylation of alpha-tubulin. How extracellular SRPX2 exerts this effect warrants further investigation and is beyond the scope of the present study. The cascade of signalling events from extracellular SRPX2 to tubulin acetylation is likely to be pluriform and very complex. Indeed, SRPX2 has numerous possible partners in the extracellular matrix and at the plasma membrane (Royer-Zemmour et al., 2008) and might influence neuronal migration through several different and non-exclusive pathways. For instance, previous studies have shown that SRPX2 modifies the phosphorylation of focal adhesion kinase (FAK) (Tanaka et al., 2009), which plays a well-known role in cell migration and participates in microtubule stabilization (Palazzo et al., 2004). Focal adhesion kinase activity, in turn, can be modulated by the urokinase plasminogen activator receptor uPAR (Tang et al., 1998), which is a SRPX2 receptor (Royer-Zemmour et al., 2008). Also, whether the effects of SRPX2 on tubulin acetylation could be influenced by uPAR, depended on the cell line that was analysed here. Not exclusively, it was also recently proposed that SRPX2 is a chondroitin sulphate proteoglycan (CSPG) (Tanaka et al., 2012); CSPGs are prominent...
components of the CNS and can influence neuronal migration (Carulli et al., 2005). Hence and in addition to its influence on tubulin acetylation, SRPX2 very likely has other functions, some of which might also interfere with cell polarity and migration. Nevertheless and despite this inherent complexity, successful ex vivo and in vivo rescues of the migration phenotype caused by Srpx2 silencing were obtained with tubacin, a specific inhibitor of tubulin deacetylase HDAC6. Whereas an acetylation-independent effect of HDAC6 inhibition on microtubule dynamics (Zilberman et al., 2009) or on other molecular targets (Valenzuela-Fernandez et al., 2008) cannot be ruled out, the efficacy of tubacin indicated that SRPX2 very likely influences neuronal migration at least partly through its action on alpha-tubulin acetylation. As a matter of fact, the phenotype caused by Srpx2 silencing recalled the
Short maternal tubacin treatment *in utero* prevents the postnatal neuronal spontaneous epileptiform activity in neocortical slices. Whole-cell voltage clamp recordings from individual neurons were performed in acute neocortical brain slices taken from post-natal Day 14 (P14) rats electroporated *in utero* at embryonic Day 15. CTL = control electroporation. 5'shSrpx2: Srpx2 silencing. 5'shSrpx2 + Tubacin: Srpx2 silencing treated with tubacin. (A–C) Representative traces of glutamatergic and GABAergic spontaneous activities recorded at membrane potentials −70 mV (spontaneous excitatory postsynaptic current) and 0 mV (spontaneous inhibitory postsynaptic current).
Mec-17 (Atat1) acetyltransferase gene knock-down model (Li et al., 2012), and the migration defects caused by Srpx2 knock-down could indeed be significantly rescued in vivo by Mec-17 overexpression. Also, the data obtained here with the two mutant SRPX2 proteins indicate that an impairment of alpha-tubulin acetylation might participate in the related human phenotypes. Generally, alpha and beta-tubulins, microtubule-regulating proteins including EFHC1, LIS1 and DCX, and tubulin modifiers such as members of the Elongator complex, play crucial roles in the development of the brain cortex and in its pathology in humans, including polymicrogyria (Creppe et al., 2009; de Nijs et al., 2009; Jaglin and Chelly, 2009; Wynshaw-Boris et al., 2010; Manzini and Walsh, 2011). Notably, Elongator is a multi-tasking complex of six subunits that is involved in the development of the cerebral cortex and in neuronal migration, and has intrinsic lysine acetyl-transferase activity (Creppe et al., 2009).

Whereas no pathogenic mutation was detected previously in SRPX2 in typical, so-called benign Rolandic epilepsy (Roll et al., 2006), or in the ELP4 Elongator subunit gene as done here in a series of 36 patients with various related and more severe disorders of the Rolandic epilepsy spectrum (Rudolf et al., 2009) (data not shown), one study has reported on the possible genetic association of ELP4 with increased risk to Rolandic epilepsy (Strug et al., 2009). Moreover, genomic duplications encompassing tubulin deacetylase gene HDAC6 were detected in patients with intellectual disability, speech delay, seizures and Rolandic-like focal electroencephalographic paroxysms (Giorda et al., 2009). The attenuation of a developmental neuronal migration disorder and of subsequent seizure susceptibility has already been obtained in vivo in a rat model of Dcx knock-down by vector-based inducible re-expression rescue experiments at early post-natal stages (Manet et al., 2009). Apart from epilepsy, motor behaviour manifestations could be prevented in a Lis1+−/− mouse model of neuronal migration disorder by prenatal injections of calpain inhibitor to the pregnant mice (Yamada et al., 2009). In the present model of Srpx2 silencing, the post-natal epileptiform consequences were prevented by maternal drug delivery during pregnancy; indeed, tubacin significantly improved the positioning of the Srpx2-silenced neurons, and this was accompanied with functional neuronal network recovery, at least at post-natal Day 14 and according to the recordings done here. The reason why a minor subset of the littermates did not apparently respond to tubacin, at least anatomically at embryonic Day 20, is unknown. Variability in tubacin transfer across the placental or blood–brain barriers cannot be excluded. Indeed, the inherently-transient biochemical effects of tubacin varied among embryos. Importantly also, administration of tubacin during a short time-period was sufficient enough for efficient prevention. Such a transient tubacin treatment led to complete rescue at the morphological and electrophysiological levels in the majority of cases, whereas significant, albeit less-pronounced rescue was obtained in vivo with vector-based re-expression of Mec-17 acetyl transferase. This suggests that the level and/or the duration of increased acetylation likely influences the efficacy of the treatment. It is noteworthy that our drug-based protocol did not yield any obvious dramatic adverse effect either in the pregnant rat or in its progeny, at least until post-natal Day 14. This is also in line with the viability and normal development of Hdac6 knock-out mice (Zhang et al., 2008). No gross side effect on cell proliferation or migration of tubacin as injected here, was observed; this apparent harmlessness might well be related to the very transient and soft features (two shots only) of the therapy. Obviously other or more subtle adverse effects cannot be excluded; as an example, the mitotic index of neural cell progenitors might be modified after acute Hdac6 inactivation in the developing brain cortex (Li et al., 2011). Also, one important challenge will involve expanding the present findings to other models of neuronal migration disorders, and testing other protocols and drugs that target microtubules, such as taxol-related molecules.

Histone deacetylase inhibitors that target tubulin (Schemies et al., 2009) have been proposed for the development of new therapies in human diseases (Kazantsev and Thompson, 2008) and have already been employed post-natally in animal models for neurological disorders (d’Ydewalle et al., 2011). Generally the prevention or attenuation of brain developmental defects should be carried out as early as possible, before the long-term clinical consequences appear later in life, and before the developmental window for such an intervention closes—hence before birth in humans. As future progress in developmental neuroimaging (Glenn and Barkovich, 2006; Studholme, 2011) and in pre-natal genetic diagnosis (Bianchi, 2012) can be anticipated, the successful in utero prevention of the post-natal epileptiform consequences of a neuronal migration disorder by a drug targeting tubulin deacetylase, as obtained here in a rat model, might be of broad putative interest given the known convergence of multiple neuronal migration pathways and disorders on alpha-tubulins.

**Figure 7** Continued

Current, respectively, from electroporated (red) and neighbouring non-electroporated (black) neurons in brain slices from control (A), 5′shSrpx2 (B) and 5′shSrpx2 + tubacin (C) rats. In (B) individual bursts (highlighted, grey boxes) are shown on the right at an extended time scale. Recordings were made simultaneously from two neurons. Note that some bursts are synchronized between two cells. (D) The increase in the number of bursting neurons caused by Srpx2 silencing was prevented by tubacin treatment. The numbers of neurons with spontaneous excitatory postsynaptic current (top) and spontaneous inhibitory postsynaptic current (bottom) interictal-like bursts are presented. Fisher’s exact test, two-tailed, Bonferroni correction. (E, F, H and I) The increase in values of maximal amplitudes and charge transfer (charge) of spontaneous excitatory postsynaptic current (E and F) and spontaneous inhibitory postsynaptic current (H and I) bursts caused by Srpx2 silencing, was prevented by tubacin treatment. The cumulative probabilities of maximal amplitudes (E and H) and charge transfer (F and I) of individual spontaneous excitatory postsynaptic current and spontaneous inhibitory postsynaptic current bursts are shown. CTL = black; 5′shSrpx2 = red; 5′shSrpx2 + Tubacin = blue. (G) The increase in spontaneous excitatory postsynaptic current integral burst charge densities (CD) seen in silencing condition was prevented by tubacin treatment. Kruskal-Wallis test, post hoc Dunn’s multiple comparison test. (J) No significant difference in spontaneous inhibitory postsynaptic current integral burst CD between control, silencing and treatment conditions. Summary of statistics is in Supplementary Table 1.
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Supplementary material

Supplementary material is available at Brain online.

References

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