Lipoprotein receptor loss in forebrain radial glia results in neurological deficits and severe seizures

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Abstract
The Alzheimer disease-associated multifunctional low-density lipoprotein receptor-related protein-1 is expressed in the brain. Recent studies uncovered a role of this receptor for the appropriate functioning of neural stem cells, oligodendrocytes, and neurons. The constitutive knock-out (KO) of the receptor is embryonically lethal. To unravel the receptors’ role in the developing brain we generated a mouse mutant by specifically targeting radial glia stem cells of the dorsal telencephalon. The low-density lipoprotein receptor-related protein-1 lineage-restricted KO female and male mice, in contrast to available models, developed a severe neurological phenotype with generalized seizures during early postnatal development. The mechanism leading to a buildup of hyperexcitability and emergence of seizures was traced to a failure in adequate astrocyte development and deteriorated postsynaptic density integrity. The detected impairments in the astrocytic lineage: precocious maturation, reactive gliosis, abolished tissue plasminogen activator uptake, and loss of functionality emphasize the importance of this glial cell type for synaptic signaling in the developing brain. Together, the obtained results highlight the relevance of astrocytic low-density lipoprotein receptor-related protein-1 for glutamatergic signaling in the

Abbreviations: GFAP, glial fibrillary acidic protein; Lrp1, low-density lipoprotein receptor-related protein-1; NSPCs, neural stem precursor cells; NMDAR, N-methyl-D-aspartate receptor; PSD-95, postsynaptic density protein 95; RT, room temperature; tPA, tissue plasminogen activator.
1 | INTRODUCTION

Low-density lipoprotein receptor-related protein-1 (Lrp1) is a member of the low density lipoprotein (LDL) receptor family and is highly expressed in astrocytes, microglia, and neurons (Auderset, Cullen, & Young, 2016). Nowadays Lrp1 is not only associated with ligand uptake, receptor mediated endocytosis, and lipoprotein transport (Herz & Bock, 2002) but also considered a matricellular receptor involved in a plethora of cellular signaling cascades (Bres & Faissner, 2019; Lillis, Van Duyn, Murphy-Ullrich, & Strickland, 2008).

In the central nervous system, the role of Lrp1 in neurons is well studied in vitro and in the adult brain. Here, Lrp1 is crucial for amyloid-beta clearance and catabolism: altered interactions of Lrp1 with neuronal amyloid-beta contribute to Alzheimer’s disease pathogenesis (Spuch, Ortoloano, & Navarro, 2012). Neuronal Lrp1 is also vital for the establishment of proper synaptic responses, including long-term potentiation (LTP) (May et al., 2004; Zhuo et al., 2000) and the regulation of calcium influx through the N-Methyl-D-aspartate receptor (NMDAR) (Qiu, Strickland, Hyman, & Rebeck, 2002). Loss of neuronal Lrp1 furthermore impacts the integrity of postsynaptic densities and leads to hyperexcitability in neuronal conditional Lrp1 mutants (Maier et al., 2013; May et al., 2004; Nakajima et al., 2013; Qiu et al., 2002).

Recent research suggests that glial Lrp1 is of equal importance for effective brain functioning: astrocytic Lrp1 mediates the uptake and metabolism of amyloid-beta, thereby becoming an essential target for Alzheimer’s disease research (B. Liu, Teschemacher, & Kasparov, 2017). Astrocyte Lrp1-mediated uptake and recycling of tissue plasminogen activator (tPA) is vital for preventing NMDAR-mediated neurotoxicity (Cassé et al., 2012). Furthermore, Lrp1 is involved in myelin phagocytosis and oligodendrocytic precursor cell differentiation and maturation (Hennen et al., 2013; Lin, Mironova, Shragar, & Giger, 2017; Safina et al., 2016). Despite progress in understanding the function of neuronal and glial Lrp1, knowledge about the role of Lrp1 in the developing brain is scarce. A hint that Lrp1 may be equally crucial in early brain development, as it is in mature circuitry, came from a study showing that the constitutive knock-out (KO) of the Lrp1 gene in mice is lethal for the embryo (Herz, Clouthier, & Hammer, 1992).

In our recent work, we unraveled that the elimination of Lrp1 in cortical neural stem precursor cells (NSPCs) in vitro leads to their altered differentiation properties including an impaired neurogenesis and reduced oligodendrocyte numbers, whereas differentiation toward astrocytes is enhanced (Hennen et al., 2013; Safina et al., 2016; Schafer et al., 2019). This raises the until now unanswered key question concerning the specific impact of Lrp1 deletion from the radial glia stem cell compartment in the living animal.

In the current study, we present a conditional KO mouse line that lacks the Lrp1 gene in radial glia cells and their progeny in the dorsal telencephalon. In order to generate these mice, we crossed Lrp1-floxed mice with a reporter line, where the Cre expression is under the control of Empty Spiracles Homebox 1 (Emx1) promoter (Iwasato et al., 2000). The expression of Emx1 in the mouse starts to become visible at embryonic day 9.5 (E9.5) and is confined to the dorsal telencephalon that gives rise to the cerebral cortex, olfactory bulbs and hippocampi. During further brain development, the Emx1 gene expression is found in radial glia and their progeny: postmitotic projection neurons of the dorsal, lateral and medium pallium that form the neocortex, piriform cortex, and hippocampi respectively as well as astrocytes and oligodendrocytes of the pallial corpus callosum and fimbria (Cecchi & Boncinelli, 2000; Gorski et al., 2002; Guo et al., 2000). Emx1 lineage cells do not give rise to GABAergic (inhibitory) interneurons and subpallial oligodendrocytes, but can contribute to the generation of excitatory cells of the amygdala and medium spiny neurons (Cocas et al., 2009; Gorski et al., 2002; Guo et al., 2000).

The Lrp1 KO mice, as described in our current work, exhibit pronounced changes in brain function, including epileptic seizures, ataxia, and an increased lateral ventricular volume. With our study, we underline a vital biological function of Lrp1 expression in the progeny of radial glia cells during early postnatal development and highlight the importance of astrocytic functionality in the establishment of appropriate synaptic connectivity. Taken together, we propose Lrp1 as a gene of interest for deciphering the mechanisms underlying the cause of seizures.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The present study was carried out in accordance with the European Communities Council Directive of September 22nd, 2010 (2010/63/EU) for care of laboratory animals and approved by a local ethics committee (Bezirksamts Arnsberg) and the animal care committee of North Rhine-Westphalia, Germany, based at the LANUV (Landesamt für Umweltschutz, Naturschutz und Verbraucherschutz, Nordrhein-Westfalen, D-45659 Recklinghausen, Germany). The study was supervised.
by the animal welfare commissioner of Ruhr-University. All efforts were made to minimize the number of mice used for this study.

2.2 | Generation of Lrp1 conditional KO mice

All animals were housed in a 12 hr light/dark cycle (lights on from 8 a.m. to 8 p.m.) with ad libitum access to food and water. Lrp1<sup>lox/lox</sup> animals (B6;129S7-Lrp1<sup>tm2Her/J</sup>; RRID:AB:IMSR_JAX:012604) (Rohllman, Gotthardt, Willnow, Hammer, & Herz, 1996) were obtained from Jackson Laboratories and kept on a C57Bl6 background (Charles River).

To generate Lrp1 telencephalic conditional KO mice, Lrp1<sup>lox/lox</sup> animals were first crossed to Emx1<sup>Cre/wt</sup> mice (Iwamoto et al., 2000). F1 Lrp1<sup>lox/lox</sup>/Emx1<sup>Cre/wt</sup> mice were subsequently crossed to Lrp1<sup>lox/lox</sup> mice. F2 Lrp1<sup>lox/lox</sup>/Emx1<sup>Cre/wt</sup> (25% of the offspring) exhibited telencephalic Lrp1 KO (see Figure S1 for mating scheme). Cre-positive Lrp1 homozygous conditional KO mice were compared to Cre-negative homozygous and heterozygous littermates in all the experiments. Mouse weight was measured starting from P12 and ending with P70 for mice that were still vital. The day of detection of a vaginal plug was taken as E0.5. For experiments, both male and female mice were used. Loss of Lrp1 expression was confirmed for all the analyzed animals.

For DNA extraction, a standard Jackson Laboratories protocol was followed. PCR primers were the following: Lrp1-for: 5'-catacccccttaaaaccccttc-3', Lrp1-rev: 5'-gcaagcttcgctacagctg-3', Emx1-for: 5'-tggtaggtcatgttgccaggttg-3', Emx1-rev: 5'-tttgggttagatatgcaggctg-3', and CRE: 5'-gcggcataaccagtgaaacagc-3'.

Both Lrp1 and Emx1/Cr genotyping reactions were run on the same program: 1. 94°C 2 min, 2. 94°C 3 sec, 3. 60°C 1 min, 4. 72°C 45 sec, 5. 72°C 5 min. Steps 1-4 were repeated 35 times. After step 5, samples were cooled down to 4°C.

For PCR products, detecting transgenes and the transgene orientation please refer to Supporting Information Figure S2A,B.

2.3 | Kaplan–Meier curve

For the generation of the Kaplan–Meier curve, survival of 50 KO and 48 wildtype (WT) mice, both male and female, was monitored. The plot was generated using the lifelines library (version 0.23.9), pandas library (version 1.0.0), matplotlib (version 3.1.2), and the Python programming language (version 3.6.8).

2.4 | Video monitoring

Three P15 KO mice (1 female, 2 males) and six P15 WT mice (3 females and 3 males) were monitored in a test cage for the occurrence of spontaneous epileptic seizures. After 2 hr of acclimation, the frequency and duration of epileptic attacks over a 1–2 week long period were video recorded with Ethovision software (Noldus, Kerpen, Germany).

2.5 | Magnetic resonance imaging

For magnetic resonance imaging (MRI), the animals were housed in groups in a temperature and humidity controlled vivarium (Scantainer Ventilated Cabinets, Scanbur A/S, Karlsunde, Denmark) with constant 12 hr light/dark cycle (lights on from 7 a.m. to 7 p.m.). The animals had ad libitum access to food and water.

Six Lrp1 KO and nine WT mice (~2 months old, both male and female) were used for the experiments. MRI images were acquired by means of a 7T horizontal bore scanner (BioSpec, 70/30 USR, Bruker, Ettlingen, Germany), using a 8.5 cm inner diameter transmit volume resonator and a planar single-loop 20 mm receiver coil. MR was conducted using the ParaVision 5.1 software (Bruker). Animals were anaesthetized using isoflurane during recordings. The respiration rate and body temperature of the animals were continuously monitored during MR scanning. 3D structural measurements were acquired at an isotropic resolution of 100 μm using a T2 weighted 3D RARE pulse sequence with the following imaging parameters: repetition time (TR) = 2.300 ms; effective echo time (TE) = 62.5 ms; RARE factor = 10; field of view (FOV) = 20 × 14 × 8 cm.

Analysis of ventricular volume was conducted using the semiautomatic segmentation function of the software ITK-SNAP 3.6 (www.itksnap.org; Yushkevich et al., 2006). Hippocampal volume was analyzed using manual segmentation with ITK-SNAP 3.6. Eleven continuous slices from the 3D images were segmented for the dorsal hippocampus, the following five continuous slices corresponded to the intermediate hippocampus and the next six continuous slices corresponded to the ventral hippocampus. One-way analysis of variance (ANOVA) was conducted using the software STATISTICA 13 (Dell, Round Rock, TX; RRID:SCR_014213).

2.6 | Behavioral analysis

2.6.1 | Gait analysis

The forepaws and hindpaws of each mouse were painted with respectively red and blue, nontoxic, water-soluble paint (Pelikan, Hannover, Germany). Each mouse was then placed at the end of a 10 cm wide, 70 cm long and 10 cm high tunnel connected with their home cage at the other end and was accessible through a hole. Each mouse underwent three pre-trials before undergoing three trials with white paper. Only one footprint pattern per mouse was scanned into the computer with a CanoScan 9000F (Canon, Tokyo, Japan). Seven steps per footprint were measured using the ImageJ software (NIH) as described previously (Klapdor, Dulfer, Hammann, & Van der Staay, 1997). In total, 8 KO (6 male, 2 female) and 10 WT (6 male, 4 female) P40 mice were tested.
2.6.2 | Vertical pole test

Motor coordination and balance skills were assessed with the vertical pole test. A 50 cm metal pole (diameter 1 cm), surrounded by electric tape to allow gripping, was placed vertically and secured to a platform. Each mouse was placed faced upward, at the top of the pole, and the latency to turn around and climb down the vertical pole was recorded. If the mouse did not complete the task within 120 sec, it fell from the pole or slid down the pole a time of 120 sec was recorded. In total, 8 KO (6 male, 2 female) and 10 WT (6 male, 4 female) P40 mice were tested.

2.6.3 | Hang wire test

The hang wire test is a simple test that assesses the balance and grip strength of mice. The mice were hung upside down on a wire screen with a 12 mm × 12 mm grid positioned 50 cm above a cage. The latency to fall into the cage was recorded. Mice that did not fall within the 60 sec trial period were removed and given a maximal score of 60 sec. In total, 8 KO (6 male, 2 female) and 10 WT (6 male, 4 female) P40 mice were tested.

2.6.4 | Rotarod

Motor coordination and balance skills were also assessed by the rotarod test (Columbus Instruments, Columbus, OH). The mice were acclimated to the rod at 4 revolutions per minute (rpm) for 1 min. The rod was then accelerated at 0.1 rpm/sec up to 40.0 rpm until all mice fell off the rod. The latency to fall and rpm at the time of the fall were recorded for each mouse. In total, 8 KO (6 male, 2 female) and 10 WT (6 male, 4 female) P40 mice were tested. Three trials per mouse were run.

2.7 | Electroencephalogram analysis

The EEG telemetry and video recordings were conducted as previously described (Bedner et al., 2015). Briefly, 7 KO and 3 WT P56 mice, both male and female, were anesthetized and placed into a stereotaxic frame. Telemetric transmitters (TA10EA-F20: DataSciences International, St. Paul, MN) were placed in a subcutaneous skin pouch made by blunt dissection under the right abdominal skin region. The monopolar leads of the transmitter were connected to stainless steel screws placed bilaterally 1.5 mm from the sagittal suture and 1.9 mm posterior to bregma. After implantation, the skull of each animal was covered with dental cement and the skin incisions were sutured. The mice were moved to clean cages and placed on individual radio receiving plates (RPC-1; DataSciences International) that captured the signal from the transmitter and transferred it via an exchange matrix to a computer equipped with the data acquisition and analysis software Dataquest A.R.T. 4.00 Gold/Platinum (DataSciences International). Additionally, the signals recorded were amplified (MPA8i, Multichannel Systems, Reutlingen, Germany; 1,000-fold, bandpass 1 Hz-5 kHz, PGA32, Multichannel Systems) and digitized (sampling rate 10 kHz, Power 1,401 analog-to-digital converter, Spike2 software, Cambridge Electronic Design Cambridge, UK). The mice were video monitored via the SeeTec video surveillance system (SeeTec, Philippsburg, Germany). The recordings were displayed, analyzed and stored on a computer with the SeeTec Office 5 software (SeeTec). The animals were recorded for 2 weeks. Afterwards the surviving mice (3 KO and 2 WT) were transcardially perfused with 4% (w/v) paraformaldehyde (PFA), the brains were carefully isolated, postfixixed overnight at 4 °C and processed for immunohistochemistry.

Analysis of the EEG data series (3 KO and 3 WT, all male) was carried out using the standard Fourier transformation in order to analyze the distribution of frequencies within seizure free regions of both WT and KO animals and regions of higher excitability in the KO animals. Two first days of EEG recordings of all animals were excluded from the analysis, as first hours of recordings are usually less stable. The seizure regions were extracted based on simultaneous visual inspection of the EEG signal and video monitoring. All regions that showed the typical form and a presence of a postictal depression were extracted as seizures. The EEG spectrum was obtained based on the Fourier transform of the EEG autocorrelation function of the extracted regions according to \( a(\omega) = \frac{1}{n} \sum_{i=0}^{n-1} e^{-\omega i} c(t) c(t + \omega) \), with \( n \) being the normalization factor and \( c(t) \) the autocorrelation function of the EEG data series \( c(t) \). The analysis utilized zero-padding and a window function of the Hann-type.

2.8 | Patch-clamp recordings

KO and WT mice aged P13-15 and P28-30, both male and female, were deeply anesthetized with isoflurane and then decapitated. The brains were rapidly removed and placed in ice cold cutting medium containing: 87 mM NaCl, 2.4 mM KCl, 1.3 mM MgSO4, 0.5 mM CaCl2, 26 mM NaHCO3, 1.25 mM NaH2PO4, 2 mM D-Glucose. Transverse hippocampal slices (350 μm) were cut on a Vibratome (VTS1000, Leica, Solms, Germany). The slices were incubated in a holding chamber with cutting media at 35 °C for 30 min. After incubation, the slices were transferred to a recording chamber positioned in the fixed-stage of an upright BX51WI microscope (Olympus, Tokyo, Japan). During the experiments, the slices were held under constant perfusion (flow rate 1.5–2 mL/m) with oxygenated artificial cerebrospinal fluid (aCSF) composed of: 125 mM NaCl, 3 mM KCl, 1.3 mM MgSO4, 2.5 mM CaCl2, 26 mM NaHCO3, 1.25 mM NaH2PO4, 13 mM D-Glucose. The temperature in the recording chamber was kept at 30 °C with the help of the Badcontroller V device (Luigs & Neumann, Ratingen, Germany).

Whole cell patch-clamp recordings were performed on hippocampal pyramidal cells of the Cornu Ammonis 1 (CA1) region using visually identified cell bodies under infrared illumination. The borosilicate glass recording pipettes, used for recordings, were filled with an intracellular solution comprising: 97.5 mM potassium gluconate, 32.5 mM KCl, 5 mM triethyleneglycoldiaminetetraacetic acid (EGTA), 10 mM...
2-(4-[2-hydroxyethyl]piperazin-1-yl)ethanesulfonic acid (HEPES), 1 mM MgCl₂, 4 mM Na₂ATP (pH = 7.3; 290 mOsM). Recordings were performed in the current-clamp mode using an amplifier (EPC10 USB, HEKA Electronic, Lambrecht/Pfalz, Germany). The data were subjected to low-pass filtering at 2.9 kHz and digitized at 10 kHz. The FITMASTER software (HEKA Electronic) was used for offline analysis. During the experiments, the cells were allowed to stay at their own resting potential. Resting membrane potential was determined as the mean value recorded during a continuous period of 60 sec. Input resistance was calculated from the slope of the linear fit of the relationship between the change in membrane potential (ΔV) and the intensity of the applied current (between −60 pA and +60 pA) during 600 ms. Membrane time constant was measured during the application of a square current −60 pA, duration 600 ms. To analyze the action potential threshold, a square current, duration 600 ms, was applied from the resting potential levels to 300 pA with 5 pA steps. The current amplitude necessary to evoke an action potential from the resting membrane potential was determined as a threshold current (in pA). Firing frequency rate was calculated as the number of spikes triggered by application of square current pulses (in 50 pA steps, duration 1 sec) in the range of 50–300 pA. Voltage sag ratio was analyzed after injection of a hyperpolarizing current from −300 pA to the resting potential (50 pA steps, duration of 1 sec).

### 2.9 Antibodies and nuclear markers

Primary antibodies: Actin (mouse; Western blot [WB]: 1:5,000; clone C4/actin, 612.657, BD Transduction Laboratories, Erembodegem, Belgium; RRID:AB_399901), ALDH1L1 (rabbit; WB: 1:500; ab87117, Abcam, Cambridge, UK; RRID:AB_10712968), GFAP (mouse; IHC: 1:500, rabbit; IHC: 1:500, WB: 1:2,000; clone G-A-5, G3893, Abcam, Cambridge, UK; RRID:AB_10712968), GFAP (mouse; IHC: 1:100, clone A60, MAB 377, Millipore; RRID:AB_10695722), GS (mouse; WB: 1:2,000; clone GS-6, D44E2, 5684, Cell Signalling Technology, Frankfurt am Main, Germany; RRID:AB_10695722), GLAST (guinea pig; EAAT1, WB: 1:1,000; clone DM1A, T9026, Sigma-Aldrich; RRID:AB_94911), NeuN (mouse; IHC: 1:100, clone A60, MAB 377, Millipore; RRID:AB_2314891), Olig2 (mouse; IHC 1:300, clone 211F1.1, MABN50, Millipore; RRID:AB_10807410), R2C (mouse; IHC: 1:200; a kind gift provided to M. Götz by Leprince, Sox-9 (rabbit; IHC: 1:1000; AB5535, Millipore; RRID:AB_2239761), Vimentin (mouse; IHC: 1:200, clone LN-6, Sigma-Aldrich; RRID:AB_261856).

Secondary antibodies: AlexaFluor 488-conjugated AffiniPure goat anti-mouse IgG + IgM (IHC: 1:500, ICC: 1:300; 115–545-044, Dianova, Hamburg, Germany; RRID:AB_2338844), Cy3-conjugated AffiniPure goat anti-rabbit IgG (IHC/ICC: 1:500; 111–165-045, Dianova; RRID:AB_2338003), Peroxidase-conjugated AffiniPure goat anti-mouse IgG + IgM (WB: 1:7,500; 115–035-068, Dianova; RRID:AB_2338005), Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (WB: 1:7,500; 111–035-144, Dianova; RRID:AB_2307391), Biotin-SP-conjugated AffiniPure goat anti guinea pig IgG (IgG [H + L], WB: 1:10,000; 106–065-003, Dianova; RRID:AB_2337410), Peroxide-conjugated Streptavidin (WB: 1:20,000; 016–030-084, Dianova; RRID:AB_2337238).

Nuclear markers: DAPI (4',6-diamidino-2-phenylindole, IHC: 1:1000; D9564, Sigma-Aldrich), HOECHST (bis Benzimide H 33258; ICC: 1:50,000; 32,883-25MG, Sigma-Aldrich), Topro3 (IHC: 1:500; 642/661, Life Technologies, Breda, Germany).

### 2.10 Western blotting

For analyzing the protein expression levels, P7, P14, P28, and P56 animals were sacrificed by cervical dislocation. The brains were frontal- and coronal-cut at 200 μm using a vibratome (VT 1000S, Leica). The cortex and hippocampus were dissected independently, immediately frozen in dry ice and stored at −70°C until lysis. The tissue was lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris–HCl (pH ~ 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM EGTA, 1% (v/v) 4-Octylphenol polyethoxylate (Triton X-100), 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 140 mM NaCl, all Sigma-Aldrich) supplemented with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/μL Aprotinin (all Roche) and homogenized with the help of a TissueLyser LT (Qiagen, Hilden, Germany). The protein concentration was assessed with the Pierce BCA Protein Assay Kit or Qubit (both Thermo Fisher Scientific, Waltham, Pittsburgh, PA). Equal levels of protein were loaded onto SDS-polyacrylamide gels and run in Mighty Small Electrophoresis Units (Bio-Rad, Munich, Germany).

Afterwards, the proteins were transferred to methanol-activated polyvinylidene difluoride (PVDF) membranes (Roth, Karlsruhe, Germany) in SDS-transfer buffer using a semidry transfer chamber (Bio-Rad). The membranes were blocked with 5% (w/v) low fat milk powder in Tris buffered saline (TBS) with Tween-20 (TBST) for 1 hr at room temperature (RT). The primary antibodies were applied in the
blocking solution overnight at 4°C. The following day, after washing steps with TBST, the membranes were incubated with horse-radish peroxidase (HRP)-conjugated secondary antibodies in the blocking solution for 1 hr at RT, followed by TBST and TBS washing steps. The signal was developed with a Clarity Western ECL Chemiluminescence Kit (Bio-Rad). Chemiluminescence images were acquired using the MicroChem Chemiluminescence-Reader (Biostep, Burkhardtsdorf, Germany) and protein density analyzed with TotalLab Quant (Cleaver Scientific Ltd, Rugby Warwickshire, UK). Western blots were analyzed by comparing the band intensity of the protein of interest normalized to the band intensity of α-tubulin or actin.

### 2.11 Cortical neuron-enriched culture

Embryos were sacrificed at E14.5 and the cortex of each littermate was dissected and processed separately. After dissociation 1 × 10^5 cells from each individual embryo were plated on 15 µg/mL poly-ornithine (Sigma-Aldrich) coated 6 cm culture dishes or 1 × 10^3 cells on 15 µg/mL poly-ornithine coated 12 mm cover slips in the differentiating medium (1:1 Dulbecco modified Eagle’s minimal essential medium (DMEM)/F12, 0.2 mg/mL L-glutamine (all Sigma-Aldrich), 100 U/mL Penicillin, 100 U/mL Streptomycin, 2% v/v B27 (all Life Technologies), 1% v/v fetal calf serum (Invitrogen, Karlsruhe, Germany)). Half of the medium was changed every 5 days. After differentiation for 14 days, the cells were subjected to the surface biotinylation experiments.

### 2.12 Cortical astrocyte-enriched culture

P2-3 pups were sacrificed and the cortex of each littermate was dissected and processed separately. The dissected cortices were immediately put in Hank’s Balanced Salt Solution (HBSS) with Ca^2+ and Mg^2+ ions (Life Technologies) and subsequently digested with HBSS containing 30 U/mL papain (Worthington, Lakewood, NJ), 24 mg/mL stock L-Cysteine (Sigma-Aldrich), and 4 mg/mL stock with HBSS containing 30 U/mL papain (Worthington, Lakewood, NJ), 24 mg/mL stock L-Cysteine (Sigma-Aldrich), and 4 mg/mL stock

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5 min), re-suspended and counted. For tPA uptake experiments and ICC, 25 × 10^3 astrocytes were plated on 100 µg/mL poly-lysine (PDL, Sigma-Aldrich) coated 4-well culture dishes (Greiner, Frickenhausen, Germany).

### 2.13 tPA in vitro uptake

The experiments were performed as described, with minor changes (Cassé et al., 2012). Briefly, after 14–16 days in culture, plated WT and KO cortical astrocytes were washed with DMEM (Life Technologies), followed by incubation with either 140 nM Fluorescein isothiocyanate (FITC) conjugated-tPA (tPA-FITC, ab92679, Abcam), 140 nM Albumin-FITC (ab8030, Abcam) or DMEM at either 37°C or 4°C for 20 min. Afterwards the cells were washed with PBS and submitted to the immunocytochemical protocol.

### 2.14 Cell surface biotinylation

WT and KO primary cortical neurons, grown in 60 mm dishes, were washed 2 times with ice-cold PBS, followed by incubation with 1 mg/mL Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) in ice-cold PBS for 40 min at 4°C. Afterwards the cells were washed 4 times with ice-cold 100 mM glycine solution in PBS, scraped of the plate and lysed in RIPA buffer with 1 mM PMSF (Roche) and 1 µg/mL Aprotinin (Sigma-Aldrich). Protein concentration was assessed using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and equal levels of protein (400–800 µg) were incubated with 20 µL of Pierce NeutrAvidin Agarose in 500 µL of RIPA buffer with inhibitors overnight. On the next day, the beads were washed 3 times with RIPA buffer, incubated at 95°C for 10 min with protein loading buffer and used for further Western blot analysis.

### 2.15 Immunohistochemistry

P7, P14, P28 and P56 mice were transcardially perfused with 4% (w/v) PFA (Riedel-de Haen Laborchemikalien, Seelze, Germany), the brains were carefully isolated and postfixed overnight at 4°C. The fixed brains were subsequently incubated in 10% (w/v), 20% (w/v) and 30% (w/v) sucrose (Fisher Scientific, Pittsburgh, PA) (each step at least 12 hr), and frozen in Tissue Freezing Medium (Leica). 40 µm-thick sections were cut and stored at −20°C in antifreeze solution (10% 10x PO4 buffer (pH 7.0), ethylene glycol 30% (w/v), glycerol 30% (w/v), all J.T. Baker, Avantor, Arnhem, The Netherlands). Prior to staining the sections were blocked with PBS containing 0.1% (w/v) Triton X-100 (Sigma-Aldrich), and 10% (w/v) normal goat serum (005–000-121, Dianova) (PBTN) for 1 hr. Subsequently sections were incubated with primary antibodies overnight at 4°C in PBTN, washed with PBS, incubated with secondary antibodies and TOPRO-3 (Life Technologies) in PBTN, washed with PBS and mounted with Immumount (Thermo Fisher Scientific).
2.16 Immunocytochemistry

The astrocyte cultures were fixed with 4% (w/v) PFA, blocked with PBTN for 30 min and incubated with GFAP antibody for 1 hr at RT. Next, the cells were washed with PBS, incubated with a secondary antibody and Hoescht (Sigma-Aldrich) for 30 min at RT, washed with PBS and mounted with Immumount (Thermo Fisher Scientific).

2.17 Cresyl violet histological stain (Nissl stain)

Briefly, cryosections from P28 mice (40 μm-thick) mounted on gelatin coated slides, were hydrated in distilled water, dehydrated (30% Ethylen alcohol [EtOH], 50% EtOH, 70% EtOH, 80% EtOH, 90% EtOH, 100% EtOH, Xylene; 3 min/step), rehydrated (100% EtOH, 90% EtOH, 80% EtOH, 70% EtOH, 50% EtOH, 30% EtOH; 3 min/step), dipped in distilled water, incubated in Cresyl Violet (Merck) for approximately 3 min. The stained sections were washed in distilled water until clear, dehydrated (70% EtOH, 100% EtOH, Isopropanol; 3 min/step), placed in Xylene (at least 5 min) and closed with Euparal (Roth).

2.18 Image acquisition and data processing

Unless otherwise stated, before quantifications, the brightness/contrast ratio was enhanced for each image and the number of cells was calculated manually using the “cell counter” plugin in Fiji (Schindelin et al., 2012).

For the assessment of GFAP- and S100-positive cell numbers in P7, P14 and P28 mice, two sections per animal (one with a visible lateral ventricle and one with the dorsal hippocampus) were stained and both hemispheres were imaged. At least four images per cortex at the level of the somatosensory and entorhinal/piriform cortex and two images per hippocampus were taken with a laser-scanning microscope (LSM) 510 Meta (Axiovert 200M, Zeiss, Jena, Germany). The number of calculated cells was averaged for each region and each animal.

For the assessment of cortical Sox9- and Olig2-positive cells in E18.5 embryos, at least three sections per mouse and two images per hemisphere were taken with the help of the LSM 510 Meta (Axiovert 200M, Zeiss). The number of calculated cells in the cortex and corpus callosum was normalized to the cortical or corpus callosum area respectively for each image and the number of cells from each region was averaged for each animal.

For the assessment of the Sox9- and GFAP-positive cells in P14 mice, two sections (one with the dorsal and one with the ventral hippocampus) were analyzed. For each hemisphere, two images of the cortex at the level of the somatosensory and entorhinal/piriform cortex and one for the dentate gyrus (DG) of the hippocampus were obtained with the Axiozoom V16 and AxioCam 506 Mono (Zeiss). For the hippocampus, the number of calculated immunopositive cells was normalized to the DG area spanning the subgranular zone and the hilus. The number of calculated cells was averaged for each region and each animal.

For the assessment of the GFAP- and Ki67-positive cells in P14 mice, two sections (one with the dorsal and one with the ventral hippocampus) were analyzed. For each hemisphere, three images of the cortex at the level of the somatosensory, entorhinal, and piriform cortex were obtained with the Axiozoom V16 and AxioCam 506 Mono (Zeiss). The number of calculated cells was averaged for each region and each animal.

For the assessment of Sox9-, GFAP-, Ki67-, and Vimentin-positive cells in P28 mice, two sections (one with the dorsal and one with the ventral hippocampus) were analyzed. For each hemisphere, two (Sox9) to three (Vimentin, GFAP, Ki67) images of the cortex at the level of the somatosensory, entorhinal and piriform cortex and one for the DG of the hippocampus (Sox9, Vimentin, GFAP) were obtained with the LSM 510 Meta (Axiovert 200M, Zeiss). For the hippocampus, the number of calculated cells was normalized to the DG area spanning the subgranular zone and the hilus (Sox9, Vimentin), or was presented as area coverage in the hilus and granule layer of the DG (GFAP, Vimentin). For each animal, the number of calculated cells was submitted to the statistical analysis without averaging.

For the assessment of Iba-1-positive microglia numbers in P28 animals, two sections (one with the dorsal and one with the ventral hippocampus) were analyzed. For each hemisphere, two images of the cortex at the level of the somatosensory cortex and two for the hippocampus were obtained with the LSM 510 Meta (Axiovert 200M, Zeiss). The number of calculated cells was averaged for each region and each animal.

For the assessment of Nestin in P14 and P28 mice two sections (one with the lateral ventricle and one with the ventral hippocampus) were analyzed. For each hemisphere, two images of the cortex at the level of the somatosensory cortex and two for the hippocampal DG were obtained with the LSM 510 Meta (Axiovert 200M, Zeiss). The images were converted to 8-bit grayscale and a threshold was determined. Nestin fluorescence intensity and Nestin coverage in the granule layer of the DG were measured with the “Measure” command in Fiji (Schindelin et al., 2012). For each DG, six measurements were made for both Nestin fluorescence intensity and Nestin-positive area. The measurements were averaged for each animal.
For the assessment of c-fos-positive cells, 10 sections spanning the entire cerebrum were analyzed in one KO P21 mouse that suffered from an epileptic seizure and one WT littermate on the LSM 510 Meta (Axiovert 200M, Zeiss).

For the assessment of c-fos basal levels in P14 and P28 mice, two sections (one with the dorsal and one with the ventral hippocampus) were analyzed. For each hemisphere, three images of the cortex at the level of the somatosensory, entorhinal, and piriform cortex and two for the hippocampus were obtained with the Axiozoom V16 and AxioCam 506 Mono (Zeiss). For the hippocampus, the number of calculated positive cells was normalized either to the hippocampal area or to the area of the DG. The number of calculated cells was averaged for each region and each animal.

For the assessment of the number of neuronal cells in P28 mice, two sections (one with the dorsal and one with the ventral hippocampus) were analyzed. For each hemisphere, two images of cortical layers IV-VI at the level of the somatosensory cortex were obtained with the LSM 510 Meta (Axiovert 200M, Zeiss). The cortical neuronal cell number was normalized to the cortical area and averaged for each animal.

For the assessment of hippocampal integrity in P28 mice, the number of TOPRO-3-positive cell nuclei in images obtained with the LSM 510 Meta (Axiovert 200M, Zeiss) was counted in the molecular layer, hilus, CA1 and CA3 of the hippocampus. For the DG, the thickness of the granule layer was additionally measured at 10 random positions per section. For all, at least two sections per animal per area were analyzed. The area of each region was measured using the free hand selection tool in Fiji (Schindelin et al., 2012). The cell number/layer thickness was averaged for each animal.

For the assessment of cerebral cortex thickness in P28 mice, tissue sections were stained with cresyl violet. Images were taken with the Axioskop (Zeiss). Ten sections were analyzed per mouse and cortical thickness was measured at six positions using the free hand selection tool in Fiji (Schindelin et al., 2012). The cortical thickness measurements were averaged for each animal.

For the preliminary assessment of lateral ventricle size in P28 mice, tissue sections were stained with cresyl violet. Images were taken with the Axioptot (Zeiss). Three sections were analyzed per mouse and ventricular size was measured with the free hand selection tool in Fiji (Schindelin et al., 2012). The lateral ventricle size was averaged for each mouse.

For the assessment of tPA levels in the hippocampus and cortex of P7, P14 and P28 mice, the images were obtained using the LSM 510 Meta (Axiovert 200M, Zeiss) and a 63× objective (Plan Apochromat, 1.4 Oil DIC). Two sections per animal (one with the dorsal hippocampus and one with the ventral hippocampus) were imaged. Regions of interest (ROIs) included cortical layers 1–3 and 4–6, hippocampal CA1, CA3 and DG. Images were subjected to background subtraction (rolling = 50) and the fluorescence intensity for each image was calculated using Fiji (Schindelin et al., 2012). The measured intensities were summed up for each ROI and averaged for each animal.

For the tPA uptake quantifications, 6–8 images per astrocyte culture were obtained randomly with the help of a fluorescent microscope (Axioskop, Zeiss). Before analysis, channels were split for each image in Fiji (Schindelin et al., 2012). The area of astrocytic coverage visualized with the help of GFAP was traced and copied onto the FITC channel with tPA puncta. Such ROIs were further submitted to background subtraction (rolling = 50) and analyzed with Fiji. The number of detected tPA puncta on the images was normalized to the area of astrocytic coverage and is presented as mean tPA uptake values for each animal.

### 2.19 Experimental design and statistical analysis

All measurements were taken from distinct samples. No sample was excluded from the IHC analysis. For the ICC analysis of astrocytic tPA uptake, astrocytes from one WT animal were excluded due to low cell quality. For Western blot experiments, in some cases, samples had to be excluded when lacking an actin or tubulin signal. The behavioral experiments as well as all data analysis were blinded. The normality and variance of data distribution was tested between groups being statistically compared via the Kolmogorow–Smirnow test.

For immuno-histo(cyto)chemistry and Western blot experiments, as the observed distributions deviated significantly from normality in most of the cases, all conditions were compared with the aid of the nonparametric Mann–Whitney U test (MW). Other experiments were subjected to one-way ANOVA, two-way ANOVA or two-way repeated measures ANOVA (rmANOVA) followed by the post-hoc Duncan tests for multiple comparisons.

Statistical tests and diagrams were made in R v. 3.0.2 (R Core Team (2013). R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/) or STATISTICA 13. Data in boxplots are presented as: center line, median; box limits, upper (75%) and lower (25%) quartiles; whiskers, minimal and maximum values excluding outliers. Data in all other formats are presented as mean ± SE or SD. F values and degrees of freedom for all ANOVAs and W values for MW tests, are included in the figure legends. Significance levels: *p < .05; **p < .01, and ***p < .001.

### 3 RESULTS

#### 3.1 Generation of Emx1Cre-Lrp1 conditional KO mice

We generated telencephalon-specific Lrp1 mutant mice by crossing Lrp1-floxed mice with a Cre reporter line (Figures S1 and S2A-B), where the Cre expression is under the control of Empty Spiracles Homebox 1 (Emx1) promoter (Iwasato et al., 2000). Emx1 expression is found as early as embryonic day 9.5 (E9.5) in the mouse and is restricted to radial glia cells of the dorsal telencephalon (Cecchi & Boncinelli, 2000). The telencephalic specificity of the Emx1-Cre line has been extensively shown by various studies (Cappello et al., 2006, 2012; Iwasato et al., 2000; Schmid et al., 2014). In our...
model, the crossing of Lrp1\textsuperscript{flox/flox} with Emx1-Cre mice resulted in a stable and consistent Lrp1 deletion in the dorsal telencephalon, as seen in lysates of acutely isolated cortical cells from E12.5-E13.0 embryos (Figure 1a), tissue sections of P14 mice (Figure 1d) and hippocampal and cortical lysates of P56 mice (Figure 1f). The remaining Lrp1 signal visible in the KO cortical and hippocampal tissue lysates in Figure 1f originates from the presence of Lrp1-expressing cells not belonging to the Emx1 lineage, like microglia (Marzolo, von Bernhardi, Bu, & Inestrosa, 2000). As anticipated from the usage of the Emx1-driven Cre line, Lrp1 was successfully lost from radial glia cells (Figure 1b–c) and their progeny, including neuronal and glial cells (Figure 1e). The restriction of Lrp1 deletion to the dorsal telencephalon is appreciated by comparison of the cortex, hippocampus, striatum and ventral midbrain in P14 and P28 mice (Figures 1d,e and S2C). While Lrp1 levels in the WT and Lrp1\textsuperscript{flox/flox} Emx1\textsuperscript{Cre/wt}, shortly Emx1Cre-Lrp1\textsuperscript{−/−} (KO mice) remain similar in the striatum (Figure 1d,e) and the ventral midbrain (Figure S2C), they are prominently reduced in the cortical and hippocampal region in the KO mice (Figures 1d,e and S2C). Given that the full Lrp1 gene KO is lethal for the embryos, the restriction of Lrp1 gene deletion to the dorsal telencephalon increased the chances of embryonic survival of Lrp1 KO mice. Compared to the available Lrp1 KO models (Q. Liu et al., 2010; May et al., 2004), our animals showed pronounced body weight loss during postnatal development (Figure 1g). The vast majority of the Emx1Cre-Lrp1\textsuperscript{−/−} mice died between 3 and 6 weeks of age (postnatal day 21–42 (P21-42)), showing a dramatic reduction in survival (Figure 1h).

3.2 | Emx1Cre-Lrp1 KO animals exhibit severe epileptic seizures

To determine the cause behind the early lethality of the Lrp1 KO mice, we monitored the mice daily, starting from P7. Upon handling of the KO mice, we observed seizure episodes, as early as P19 that corresponded in their severity to stage 4 and 5 of Racine’s classification (Racine, 1972). Video monitoring of P15-28 mice confirmed that seizures indeed contributed to the premature mortality of the gross number of Emx1Cre-Lrp1\textsuperscript{−/−} mice. For example, the P19 KO mouse shown in Supporting Information Videos S1 and S2 experienced a severe seizure episode (Supporting Information Video S1) that resulted in hindlimb paralysis (Supporting Information Video S2) and death shortly afterwards. To confirm the epileptic nature of the seizure episodes and determine whether surviving, adult mice continued to suffer from seizures, electroencephalogram (EEG) recording analysis was performed. The EEG confirmed the presence of spontaneous generalized seizure episodes in all analyzed P56 Emx1Cre-Lrp1\textsuperscript{−/−} mice, while no abnormal activity was seen in WT littermates (Figures 2a–d and S3). On average, the KO mice suffered daily from at least one, tonic–clonic seizure episode with rearing, head nodding, Straub tail and facial movements (Figure 2d and Supporting Information Video S3). To gain a better understanding of the seizure properties, Fourier transformation of the EEG recordings was implemented. When compared to either the WT or KO basal activity traces, the KO seizure traces demonstrated a shift toward higher intensities and lower frequencies, highlighting synchronized activity (Figure 2b,b′,c). Although in the quantifications, both the KO seizure and the KO basal traces showed increased intensities, the frequency distribution in the WT and KO basal traces was very similar. In contrast, between WT basal and KO seizure traces there was no overlap (Figure 2b,c). This in turn suggests that not only seizure activity but also basal activity in the KO is altered.

On the cellular level, a reason behind the development of recurrent, unprovoked seizures in our mutant, may be a shifted neuronal excitation to inhibition balance. To clarify whether hyperexcitability neurons are present before seizure onset or coincide with it, patch-clamp ex vivo recordings from pyramidal cells in the hippocampal Cornu Ammonis 1 (CA1) of P14 and P28 WT and KO animals were performed. The recordings revealed that only cells in the hippocampus of P28 KO mice showed changes in their intrinsic properties indicating that hyperexcitability and epileptic activity arise simultaneously after P14 (Figure 2e–i and Table 1). The detected impairments included decreased action potential thresholds and increased action potential firing rates (Figure 2f,h), all hallmarks of a hyperexcitable phenotype. In support of our findings, subsequent histology revealed that the upregulation of proliferating cells in the hippocampal dentate gyrus (DG), which is typical for acute phase epilepsy (Gu, Li, Shang, Hou, & Zhao, 2010; Sankar, Shin, Liu, Katsumori, & Wasterlain, 2000), only occurred in P28 KO mice (Figure S4A,B). To further confirm increased excitability and determine affected regions, a P21 KO mouse that suffered from a seizure episode was transcardially perfused 2 hr after seizure onset together with a WT littermate. In order to detect active cells an antibody against the proto-oncogene c-fos was used. C-fos is an established marker for the detection of generalized seizure episodes as its basal expression is low in neurons and becomes only transiently expressed after synaptic activation occurs (Dragunow & Robertson, 1987; Sagar, Sharp, & Curran, 1998). In agreement with patch-clamp results, prominent c-fos staining was present in the hippocampus and cortex of the KO animal, confirming that seizures are concurrent with neuronal hyperexcitability (Figure S4C,D, respectively). The analysis of basal c-fos levels in P14 KO mice revealed that the number of cortical c-fos cells was mildly increased, indicating an ongoing buildup of hyperexcitability (Figure S5A–C). In the hippocampus, the number of c-fos cells was not altered (Figure S5D–F). At P28, cortical c-fos cell numbers were not altered (Figure S6A–C). While the total c-fos cell number in the hippocampus was not changed (Figure S6D,E), a significant decrease was present in the hippocampal DG (Figure S6D,F). As shown in recent studies, hippocampal c-fos expression is biphasic: it increases after acute neuronal activity, but decreases upon chronic activation of neurons (Calais et al., 2013; Corbett et al., 2017). The acute increase and basal decrease of c-fos cells observable especially in the hippocampus of P28 KO mice indicates therefore the beginning of a chronic epileptic phenotype in juvenile mice.

In sum, the combined results of our EEG, patch-clamp and histology findings put forward that increased hyperexcitability and resulting severe epileptic activity emerge as early as 3 weeks postnatally in
FIGURE 1  Emx1Cre-Lrp1−/− mice display consistent loss of Lrp1 in radial glial cells and progeny, decreased weight and reduced survival. (a) Western Blot of acutely isolated E12.5-E13.0 cell lysates from the cortices of WT, KO and heterozygous embryos. (b and c) Representative images of the somatosensory cortex of E18.5 WT (b, b′, b″) and KO (c, c′, c″) embryos visualizing loss of Lrp1 signal (gray) along radial glial fibers (magenta) (white arrows in b′, b″ and c′, c″). (d) Representative images of P14 WT and KO mice showing the restriction of Lrp1 deletion to the dorsal telencephalon. (e) Higher magnification views of the P14 cortex, hippocampus and striatum. White arrows on images of the cortices and hippocampi point to astrocytes. The white arrowheads indicate presumed neuronal cells. (f) Representative Western blots for Lrp1 protein levels in the cortex and hippocampus of P56 WT and KO mice (MW, both W = 42, N = 7 WT, 6 KO). (g) Weight comparison of KO mice (gray line and points) with age-matched WT mice (black line and points) (N = 244 WT, 101 KO). (h) Kaplan–Meier survival curve for WT (black line) and KO (gray line) mice that were followed in their survival for the time frame of 100 days (N = 48 WT, 50 KO). Scale bars correspond to 100 μm in (b and c), 200 μm in (d) and 20 μm in (e). Data are expressed as median with interquartile ranges (f) (** indicates p < .01). CA1, Cornu Ammonis 1; cx, cortex; g, grams; GFAP, glial fibrillary acidic protein; h, hippocampus; HET, heterozygote; kDa, kilo Dalton; KO, knock-out; LRP1, low-density lipoprotein receptor-related protein-1; N, number of animals; MW, Mann–Whitney U test; P, postnatal day; RC2, radial glial cell marker-2; str, striatum; W, statistics value for the MW test; WT, wildtype; %, percent; μm, micrometer. Supporting Information is presented in Figure S2C.
FIGURE 2  Legend on next page.
Emx1Cre-Lrp1\(^{-/-}\) mice, contribute to the shortened life span and weight loss and persist in surviving mice.

### 3.3 Emx1Cre-Lrp1 KO animals exhibit ataxia and an enlarged ventricular volume

As Lrp1 mouse models show motor impairments (Q. Liu et al., 2010; May et al., 2004), we expanded the examination of our mutants by determining whether, in addition to epilepsy, also motor deficits occurred in Emx1Cre-Lrp1 KO mice. In the tail suspension test, young, P20 Emx1Cre-Lrp1\(^{-/-}\) mice promptly clasped their hindlimbs (Figure 3a), a sign of either motor excitation, or reduction in motorneuron inhibition (Q. Liu et al., 2010; May et al., 2004). To elucidate whether Lrp1 KO mice indeed suffered from motor impairments, we subjected surviving P40 mice to a battery of motor tests. The motor tests specified that Emx1Cre-Lrp1\(^{-/-}\) mice displayed an ataxic phenotype, given their impaired performance in the rotarod, pole and hangwire tests (Figure 3b–d). A major characteristic of ataxic mice is the reduction in stride length, which is compensated by an increase in stride width. Subsequent footprint analysis of our mice unraveled altered gait including a decrease in stride length and, surprisingly, stride width (Figure 3e,f). In accordance, as seen in Figure 3f, KO mice require more steps to cover the same distance.

To delineate the cellular basis of the ataxic phenotype, brain tissue sections of the dorsal telencephalon of P28 WT and KO mice were analyzed. In comparison to age-matched WT mice, we found a striking increase in lateral ventricle size (Figure S7A,B,D) and compressed appearing hippocampi in KO mice (Figure S7D). To evaluate our observations quantitatively, we subjected P56 Lrp1 KO and WT animals to a magnetic resonance imaging (MRI) analysis. We performed the MRI analysis on P56 mice, as the experimental setup was not suitable for younger animals. MRI validated significant differences in the volume of the ventricles and the dorsal hippocampus (Figure 4a–i). In particular, the lateral ventricles were significantly larger (Figure 4c), while the fourth ventricle was significantly smaller (Figure 4d). There was no visible increase in size of the third ventricle (Figure 4e). Volume analysis revealed that the dorsal hippocampus was significantly smaller in Emx1Cre-Lrp1\(^{-/-}\) mice (Figure 4f), whereas the intermediate and ventral

### TABLE 1  Comparison of membrane properties of pyramidal cells recorded from CA1 area of the hippocampus of P14 and P28 mice

| Membrane property                  | LRP \(n = 17\) | WT \(n = 19\) | \(p\)  | LRP \(n = 12\) | WT \(n = 14\) | \(p\)  |
|------------------------------------|----------------|--------------|-------|----------------|--------------|-------|
| Resting membrane potential, mV     | \(-60.71 \pm 2.08\) | \(-61.72 \pm 3.34\) | .289  | \(-65.1 \pm 2.52\) | \(-66.96 \pm 2.07\) | .049  |
| Input resistance, MΩ               | 261.47 \(\pm 71.54\) | 250.47 \(\pm 65.98\) | .634  | 174.25 \(\pm 24.49\) | 139.14 \(\pm 40.18\) | .014  |
| Membrane time constant, ms         | 17.09 \(\pm 5.06\) | 18.88 \(\pm 6.52\) | .226  | 14.35 \(\pm 2.69\) | 15.18 \(\pm 1.46\) | .394  |
| AP width half amplitude, ms        | 1.209 \(\pm 0.18\) | 1.152 \(\pm 0.15\) | .338  | 1.012 \(\pm 0.09\) | 0.96 \(\pm 0.08\) | .207  |
| AP spike amplitude, mV             | 91.55 \(\pm 7.03\) | 88.38 \(\pm 5.51\) | .139  | 95.93 \(\pm 5.65\) | 99.96 \(\pm 5.99\) | .938  |
| Threshold, pA                      | 41.47 \(\pm 26.44\) | 50.78 \(\pm 22.43\) | .268  | 72.08 \(\pm 16.57\) | 123.21 \(\pm 41.25\) | .0005 |
| Voltage sag, mV                    | 16.31 \(\pm 3.72\) | 15.59 \(\pm 5.38\) | .649  | 11.19 \(\pm 2.58\) | 6.77 \(\pm 3.14\) | .0007 |

**Note:** This is a summary table depicting data shown in Figure 2e–i. Data are presented as mean \(\pm\) SD. For statistics, one-way analysis of variance (ANOVA) was applied, for analyzing firing frequency and voltage sag ratio two-way ANOVA repeated measures was applied. \(n\) is the number of neurons, \(p\) the probability, and \(P\) the postnatal day.

### FIGURE 2  Prominent generalized seizures and altered intrinsic properties of hippocampal pyramidal cells are present in Emx1Cre-Lrp1\(^{-/-}\) mice.

(a) Representative EEG traces showing basal WT activity (top panel), basal KO activity (middle panel) and a generalized KO seizure episode (bottom panel). (b) Graphical representation of Fourier-transformed EEG frequency spectra present during WT basal activity (black line), KO basal activity (dashed black line), as well as KO seizure activity (black line with crosses). (b') A magnified view of the lower frequency spectrum. Arrow points to a distinct shift toward lower frequencies in the KO seizure activity trace (P56, \(N = 3\) WT, 3 KO). (c) Graphs visualizing the intensity distribution for frequencies between 0 and 3 Hz (MW, \(W = 0\) for WT basal vs. KO seizure and \(W = 10783527\) for WT basal vs. KO basal, \(N = 3\) WT, 3 KO). (d) Images presenting different states of a tonic–clonic epileptic seizure of a P56 KO mouse recorded in Video S3, (e–f) Intrinsic membrane properties of CA1 hippocampal pyramidal cells during early development. (e) Resting membrane potential in P14 and P28 KO mice compared to age-matched WT mice (P28, one-way ANOVA, \(F(1, 24) = 4.272\)). (f) Action potential threshold in P14 and P28 KO mice compared to age-matched WT mice (P28, one-way ANOVA, \(F(1, 24) = 16.118\)). (g) Action potential firing rates in P14 WT and KO mice. (h) Action potential firing rates in P28 WT and KO mice (two-way ANOVA, \(F(1, 24) = 0.451\)). (i) Example of action potential firing rates obtained during current application of 100 pA. Scale bars correspond to 10 mV (vertical bar), 100 ms (horizontal bar). Data are expressed as median with interquartile ranges (c) or mean \(\pm\) SE (e–h) (* indicates \(p < .05\), ** indicates \(p < .01\), *** indicates \(p < .001\), (e–h) \(N = 6\) WT, 6 KO; \(n = 19\) WT, 17 KO (P14); \(n = 14\) WT, 12 KO (P28). A.U., arbitrary units; g, grams; Hz, hertz; kDa, kilo Dalton; KO, knock-out; ms, milli second; mV, milli volt; MW, Mann–Whitney U test; N, number of animals; n, number of neurons; P, postnatal day; pA, pico-ampere; sec, second; vs., versus; W, statistics value for the MW test; WT, wildtype. Supporting Information is presented in Figure S3-6 as well as in Table 1 and Supporting Information Videos S1, S2 and S3.
Hippocampi were unchanged (Figure 4g,h, respectively; Figure 4i shows additionally a tridimensional overview of the analyzed hippocampal regions). The observed alterations in hippocampal and ventricular volume were not accompanied by changes in DG and cerebral cortex thickness (Figures S8D and S7C, respectively), nuclei number decrease in the hippocampal DG, CA1, Cornu Ammonis 3 (CA3) (Figure S8D,E) or NeuN-positive cell number decrease in the cortex (Figure S8A–C).

In summary, the presence of generalized seizures in conjunction with significant weight loss, a reduction in animal survival, ataxia and enlarged lateral ventricular volume reflect a surprisingly severe phenotype in a lineage-restricted Lrp1 model, raising questions regarding the role of Lrp1 in the establishment of proper morphology, synaptic transmission and connectivity in the developing mouse brain.

3.4 | Emx1Cre-Lrp1−/− mice display a decrease in PSD-95 and in vitro Emx1Cre-Lrp1−/− cortical neurons have altered levels of NMDAR on their surface

Seizures are caused by a deficit in adequate neuronal signaling. As indicated in Figures S7C and S8A-C, the neuronal distribution in the dorsal telencephalon was not impacted in our KO mice, which is in agreement with the results obtained for the SynapsinCre-Lrp1−/− KO
mouse model (May et al., 2004). Despite unaltered neuron numbers, we hypothesized that neuronal properties could still be changed, as shown for other Lrp1 mutant models. Neuronal Lrp1 is widely known for its interactions with postsynaptic density protein 95 (PSD-95) and NMDAR, influencing LTP and regulation of synaptic plasticity (Maier et al., 2013; May et al., 2004; Nakajima et al., 2013). As recently
shown, Lrp1 presence affects synaptic stability by decreasing PSD-95 expression and plays a role in NMDAR recycling on the neuronal surface (Q. Liu et al., 2010; Maier et al., 2013; Martin et al., 2008). Western blot (WB) analysis revealed a significant, tissue-heterogeneous decrease of PSD-95 protein levels in P28 and P56 Emx1Cre-Lrp1^{-/-} mice (Figure 5a–d), with no significant changes

**FIGURE 5** Emx1Cre-Lrp1^{-/-} mice show decreased PSD-95 levels in vivo and increased GluN1 levels in vitro. (a and b) PSD-95 levels in P28 mouse cortices (a) and hippocampi (b) (MW, A: W = 15, B: W = 19, N = 5 WT, 4 KO, three repetitions for each sample). (c and d) P56 cortical (c) and hippocampal (d) PSD-95 levels (MW, C: W = 37, D: W = 42, N = 7 WT, 6 KO, two repetitions for each sample). (e and f) NMDAR GluN1 subunit protein levels in P28 cortices (e) and hippocampi (f) (MW, e: W = 17, N = 6 WT, 6 KO, f: W = 18, N = 6 WT, 5 KO; two independent experiments). (g) GluN1 protein levels present on the surface of E14.5 WT and KO neuron-enriched cultures (MW, W = 0, N = 4 WT, 4 KO; two independent experiments). Data are expressed as median with interquartile ranges (* indicates p < .05, ** indicates p < .01). For all Western blots, representative blots are shown. E, embryonic day; GluN1, N-methyl-D-aspartate receptor subunit 1; kDa, kilo Dalton; KO, knock-out; N, number of animals; MW, Mann–Whitney U test; P, postnatal day; PSD-95, post synaptic density protein 95; W, statistics value for the MW test; WT, wildtype. Supporting Information is presented in Figure S9
proceeded to determine whether cortical and hippocampal astrocytes increased astrocyte progeny number (Safina et al., 2016), we asked considering that the in vitro KO of Lrp1 in cortical NSPCs leads to an changes in the astrocytic population

| 3.5 | Emx1Cre-Lrp1 KO mice show prominent changes in the astrocytic population |

Considering that the in vitro KO of Lrp1 in cortical NSPCs leads to an increased astrocyte progeny number (Safina et al., 2016), we asked whether neuronal deficits present in Emx1Cre-Lrp1−/− mice were accompanied by alterations in radial glia and astroglia during embryonic and postnatal development.

For the analysis of embryonic development, we chose to look at E18.5 as at this time point gliogenesis is prominent. The intensity of the radial glia cell marker-2 (RC2) at this stage was not significantly altered indicating Lrp1 loss did not cause prominent radial glia impairments (Figure 6). The analysis of the astrocytic lineage at E18.5 was performed by using oligodendrocyte transcription factor 2 (Olig2) (Woodruff, Tekki-Kessaris, Stiles, Rowitch, & Richardson, 2001; Zhou, Wang, & Anderson, 2000) and Sox9, a recently described specific nuclear marker for astrocytes outside neurogenic regions and a marker for neural progenitor cells in neurogenic regions (Sun et al., 2017). The number of Sox9-positive cells in the cortical plate, intermediate zone, subventricular zone and ventricular zone was not changed between WT and KO mice (Figure 6a–e). The number of Olig2 and Sox9-double positive cells also showed no alteration in the cortical plate, intermediate zone and subventricular zone (Figure 6f–h), but a mild increase in their number was apparent in the ventricular zone (Figure 6i). Given that we found no prominent alterations in the astroglia at E18.5, we focused on studying postnatal development of astrocytes. We examined P0 animals but found, similar to E18.5, no changes in Sox9-positive cell number in the cortices or the corpus callosum of KO mice (Figure S10A). In the next step we therefore looked at the protein levels of glial fibrillary acidic protein (GFAP) in P7, P14 and P28 mice. During neonatal development, GFAP levels remained stable (Figure S11A,B), but became significantly increased in cortices and hippocampi of P28 mice in comparison to a broad astrocyte marker aldehyde dehydrogenase 1 family member L1 (ALDH1L1) (Cahoy et al., 2008) (Figures 7a and S12A,B). This increase persisted in P56 mice (Figure 7b).

In support of the developmental upregulation of GFAP protein levels in KO animals, we found an increased number of GFAP-positive cells in the cortex and hippocampus of P28 KO mice (Figure 7f,g,j) but not in P7 (Figure S13A,C,D) and P14 KO mice (Figure 7c,d,i). To further analyze astrocyte development in neonatal and juvenile mice we used S100 calcium binding protein b (S100b), a broader astrocyte marker, the expression onset of which has been shown to occur when neocortical GFAP-positive cells lose their neural stem cell potential (Raponti et al., 2007). S100b levels were constant at P7 (Figure S13B,C,E) and at P28 (Figures 7f,h and 13G). At P14, a significant increase in cortical S100b-positive cell number (Figure 7e), but not hippocampal (Figure S13F) was observed. To confirm whether such transient S100b upregulation indicated increased astrocyte numbers and to clarify whether the upregulation of GFAP pointed to the presence of hypertrophic astrocytes (Pekny & Nilsson, 2005; Sofroniew & Vinters, 2010), we investigated in more detail astrocytic development at P14 and P28—stages before and after seizure onset, respectively.

We examined the levels of Vimentin, a type III intermediate filament protein that is both expressed in neural stem cells and considered a reactivity marker for astrocytes alongside Nestin (Liddelow & Barres, 2017). Vimentin expression appeared to be most visible in the hippocampal DG and at the level of the piriform cortex in P28 mice, supporting the presence of reactive astrocytes in seizing animals (Figure S10B). Analysis of Sox9-positive cells in P28 mice revealed that although the somatosensory cortex did not exhibit altered astrocyte numbers (Figure 8a), at the level of the entorhinal/piriform cortex, a significant increase was found (Figure 8b). Both Vimentin and GFAP-positive cell number was significantly increased in all analyzed cortical regions, however the increase at the level of the piriform cortex was most prominent (compare Figure 8d with Figure 8e). The
FIGURE 6  No alterations in astrocytic development found in E18.5 Emx1Cre-Lrp1−/− mice. (a) Representative images of Sox9 and Olig2 cells in E18.5 mice. (b–e) Number of Sox9-positive cells in the cortical plate (b), intermediate zone (c), subventricular zone (d) and ventricular zone (e) is not changed in E18.5 KO mouse embryos (MW, b: W = 80, c: W = 107, d: W = 103, e: W = 90, N = 3 WT, 3 KO). (f–i) The number of Sox9 Olig2-double positive cells in the cortical plate (f), intermediate zone (g), and subventricular zone (h) is not changed while it is mildly increased in the ventricular zone (i) in E18.5 KO mouse embryos (MW, f: W = 8, g: W = 22, h: W = 24, i: W = 6, N = 3 WT, 3 KO). (j) Representative images showing radial glial fiber intensity in the cortex of E18.5 mouse embryos (MW, W = 44, N = 3 WT, 3 KO). Scale bars in a and j represent 100 μm. Data are expressed as median with interquartile ranges (* indicates p < .05). A.U., arbitrary units; IZ, intermediate zone; KO, knock-out; MW, Mann–Whitney U test; Olig2, oligodendrocyte transcription factor 2; RC2, radial glial cell marker-2; Sox9, transcription factor Sox9; SVZ, subventricular zone; VZ, ventricular zone; W, statistics value for the MW test; WT, wildtype; #, number of cells; μm, micrometer. Supporting Information is presented in Figure S10A.
FIGURE 7  Legend on next page.
analysis of proliferating Ki67 cells and proliferating astrocytes (Ki67-GFAP-double positive cells) revealed no significant changes (Figure 8c–e).

In P14 mice, neither Sox9- or GFAP-positive cell number was changed at the level of the somatosensory cortex and entorhinal/piriform cortex (Figure 9a–f). Bulk proliferating cell and proliferating astrocyte numbers were not altered at the level of the somatosensory and entorhinal cortex (Figure 9g–l). We noted a significant increase of proliferating astrocytes, but not bulk proliferating cells at the level of the piriform cortex (Figure 9m–o).

Taken together, our analysis suggests firstly, the occurrence of altered cortical astrocyte maturation in P14 KO mice and secondly, that astrocytes located in the ventral cortical regions are impacted in our model the strongest.

Given that Vimentin immunolabeling was also found in the hippocampal DG (Figure S10B), we extended our analysis also to the dorsal and ventral DG in P14 and P28 mice. We examined Nestin, a marker for neural stem cells, but found no changes in Nestin fluorescence intensity or Nestin area coverage at P14 or at P28 (Figure S10C,D). As shown in Figure 10, a reduction in the number of Sox9-positive cells in the hilus, a decrease in the number of Sox9-positive stem cells and a prominent upregulation of Vimentin was present in both the dorsal (Figure 10a–d) and ventral (Figure 10e–h) DG. As both the dorsal and ventral DG showed similar changes we analyzed GFAP-and Vimentin-positive cell coverage in the dorsal and ventral DG together. This analysis revealed increased coverage of both markers in both analyzed regions (Figure 10i–m). A clear shift of the distribution of Vimentin- and GFAP-positive stem cell fibers was noted: while in the WT mice, these fibers spanned throughout the granule layer width, in the KO the fibers appeared shorter and thicker (red arrowheads in Figure 10i). The alterations found at P28 were prominent, but confined to this stage as in P14 mice no significant changes in stem cell fiber morphology and in the number of Sox9 and GFAP cells were obvious (Figure 11a–h).

Together, an altered number of Sox9 stem cells, increased GFAP and Vimentin numbers and changed morphology of stem cell fibers in the P28 DG indicate the ongoing development of a seizure-reactive gliosis that impacts the hippocampal neurogenic niche, as shown previously by (Muro-Garcia et al., 2019).

Reactive gliosis can be accompanied by an inflammatory response as shown for various injury models (Liddelow & Barres, 2017). The co-joint presence of reactive astrocytes and an increased number of iba-1 microglial cells has been found for a different Lrp1 mutant (Q. Liu et al., 2010). To clarify whether in our model this was also the case, the iba-1 positive cell number in the tissue of P28 mice was counted. No increase in the number of cortical or hippocampal iba-1-positive cells was detectable (Figure S11C,E–G, respectively), however iba-1-positive cells with an ameboid (active) morphology were encountered in cortical areas where the glial response was most prominent (Figure S11D). This in turn suggests that astroocyte reactivity at least in the cortex can be accompanied by mild activation of microglial cells at this stage.

Taken together, the above results indicate that Emx1Cre-Lrp1−/− mice exhibit developmental alterations in the astrocytic progeny, seen both prior to the appearance of neuronal deficits as well as coinciding with hyperexcitability and seizure emergence. Both characteristics impact on proper circuit formation and maturation in our model (Volterra & Meldolesi, 2005). As the above results suggest that glial changes are also the cause and not merely the consequence of epilepsy, we next elucidated whether astrocytic functionality was affected by Lrp1 loss. In the latter case, astrocytes would emerge as the key player explaining the different severity of phenotypes between our Lrp1 mutant and others.

### 3.6 | Emx1Cre-Lrp1 KO mice exhibit developmental variations in glutamate transporter levels

Glutamate clearance is one of the major functions of astrocytes that can be altered during epilepsy (Seifert, Schilling, & Steinhauser, 2006). Proteins that are critical for astrocytic glutamate uptake are glutamate transporters Eaat1 (GLT-1) and Eaat2 (GLAST). In the neonatal cortex and hippocampus GLT-1 levels are low, while GLAST expression is high. GLT-1 controls 90% of total glutamate uptake, its expression increases significantly during synapse formation, and is heterogeneously controlled in the cortex and hippocampus (Danbolt, 2001; Hanson et al., 2015).
FIGURE 8  Legend on next page.
Both GLAST and GLT-1 levels have been shown to be lower in the cortex than in the hippocampus. In consequence, glutamate uptake in the neonatal cortex is slower in comparison to the neonatal hippocampus (Hanson et al., 2015). Although the lack of GLT-1 leads to spontaneous seizures, while its overexpression mitigates epilepsy (Kong et al., 2012; Petr et al., 2015; Tanaka et al., 1997), more complex GLT-1 expression changes during the time course of epilepsy were discovered recently (Hubbard, Szu, Yonan, & Binder, 2016). As reduced efficiency of astrocytic glutamate uptake leads to increased glutamate presence in the extracellular space and can cause excessive neuronal activity, we were interested whether changes in glutamate transporter levels were present in our KO mice before seizure onset. As visible in Figure 12a,b, at P7 GLT-1 levels remained unchanged. A prominent increase was found in the cortex (Figure 12c) but not in the hippocampus (Figure 12d) of P14 KO mice, providing support for our S100b results (see Figure 7c,e). GLAST levels were significantly increased in the hippocampus but not in the cortex of P7 Emx1Cre-Lrp1−/− mice (Figure 12g,h). At P14, GLAST levels were unaltered in both tissues (Figure 12i,j). To elucidate whether in adult KO animals with spontaneous, chronic epileptic activity glutamate receptor levels were altered we looked at P56 mouse tissue. Here, the levels of both, GLT-1 and GLAST, were not influenced (Figure 12e,f and k,l, respectively).

Glutamine synthetase (GS) is an astrocytic enzyme in charge of the glutamate–glutamine cycle that is crucial for inhibitory synaptic transmission. It has been shown that the presence of reactive astrocytes leads to GS downregulation and disruption of neuronal glutamine stores (Ortinski et al., 2010). A malfunctioning supply of glutamine to GABAergic neurons can, in turn, disrupt synaptic signaling and result in seizures (Eid, Tu, Lee, & Lai, 2013; Liang, Carlson, & Coulter, 2006). In line with unaltered glutamate transporter levels, we detected no changes in cortical and hippocampal GS levels in P56 Emx1Cre-Lrp1−/− mice (Figure S12C), implying that the supply of glutamine for GABA replenishment is still sufficient in our animals.

Together, the above indicates that although glutamate uptake appears not to be disrupted in P28 and P56 mice, the detected transient neonatal changes in the ratio of glutamate transporters support the presence of precocious astrocyte maturation and inadequate astroglial development in the current mutant.

3.7 | In vitro Emx1Cre-Lrp1−/− derived cortical astrocytes show reduced tPA uptake capabilities

Healthy astrocytes possess the ability to influence the efficacy of synaptic responses not only by means of the clearance of extracellular glutamate, but also via tPA (Fernandez-Monreal, Lopez-Atalaya, Benchene, Leveille, et al., 2004). The serine protease, tPA, is both a potent NMDAR interaction partner, a prominent Lrp1 ligand, as well as an immediate-early gene activated by neuronal activity during long-term depression (LTD), LTP and seizures (Nicole et al., 2003; Tsirka, Guanandris, Amaral, & Strickland, 1995; Zhuo et al., 2000). In vitro astrocytes with reduced levels of Lrp1 do not internalize neuron derived tPA efficiently, resulting in elevated levels of tPA in the synaptic cleft, that affect NMDAR-mediated signaling (Cassé et al., 2012; Fernandez-Monreal, Lopez-Atalaya, Benchene, Cacquevel, et al., 2004; Fernandez-Monreal, Lopez-Atalaya, Benchene, Leveille, et al., 2004; Makarova et al., 2003). We hypothesized therefore that in our Lrp1 mutants the resultant absence of Lrp1 from the astrocytic surface may lead to increased levels of extrasynaptic tPA. This, combined with neuronal NMDAR upregulation and PSD-95 decrease, could represent a factor favoring increased excitability of glutamatergic neurons, and consequently epilepsy in our mice. To test this hypothesis, we firstly assessed the levels of tPA present in cortical and hippocampal P7, P14 and P28 Lrp1 WT and KO tissue. The results confirmed that tPA levels in the cortex and hippocampus were altered during early postnatal development in vivo (Figures 13a,b and S14). To evaluate directly whether astrocytic tPA uptake deficits were underlying tPA level changes seen in vivo, we performed a tPA uptake assay on cultured WT and KO postnatal cortical astrocytes. WT astrocytes readily and uniformly took up tPA (Figure 14a,a’) while Emx1Cre-Lrp1−/− astrocytes displayed a reduced ability to internalize tPA (Figure 14b,c). In control conditions, minor uptake occurred at 4°C for tPA, confirming that it is an active process (Figure 14d). As an internal control for the uptake, we incubated the WT astrocytes with albumin at 37°C and, as visible in Figure 14e, no albumin uptake occurred in these conditions.

Taken together, deficits in astrocytic tPA uptake capabilities and neuronal tPA protein level changes present in our model affect synaptic signaling and contribute (together with gliosis, changed astrocyte maturation, neuronal NMDAR upregulation and PSD-95 deficiency) to increased excitability.

**FIGURE 8** Emx1Cre-Lrp1−/− mice at the age of P28 show increased Sox9, GFAP and Vimentin without increase in the numbers of proliferating cortical astrocytes. (a and b) Sox9 cells at the level of the somatosensory cortex (a) and entorhinal/piriform cortex (b) of P28 WT and KO mice (MW, a: W = 11, b: W = 5, N = 3 WT, 3 KO), (c-e) GFAP, Vimentin, proliferating Ki67-positive cells and Ki67-positive astrocytes at the level of the somatosensory cortex (c), entorhinal cortex (d) and the piriform cortex (e) (MW, c: GFAP, W = 3, Vimentin, W = 14, Ki67, W = 42, Ki67 + GFAP, W = 14, d: GFAP, W = 0, Vimentin, W = 10, Ki67, W = 71, Ki67 + GFAP, W = 9, e: GFAP, W = 2, Vimentin, W = 0, Ki67, W = 54, Ki67 + GFAP, W = 11, N = 3 WT, 3 KO). Red arrowheads in (d) mark reactive astrocytes, a proliferating astrocyte is marked with a red arrow. In (e) red arrowheads mark proliferating, reactive astrocytes. The red rectangles on the schematic brain sections in each panel show which cortical region the presented images are from. For example, in panel (a) the images presented are from the somatosensory cortex at the level of the ventral hippocampus. For all, representative images are shown. Scale bars in (a–e) represent 100 μm. Data are expressed as median with interquartile ranges (* indicates p < .05, ** indicates p < .01). GFAP, glial fibrillary acidic protein; Ki67, marker of proliferation; KO, knock-out; MW, Mann–Whitney U test; Sox9, transcription factor Sox9; Vimentin, type III intermediate filament protein; W, statistics value for the MW test; WT, wildtype; μm, micrometer; #, number of. Supporting Information is presented in Figure S10B.
FIGURE 9  Legend on next page.
4 | DISCUSSION

In this study, we examined the consequences of Lrp1 gene deletion in the radial glia compartment of the mouse dorsal telencephalon. Our results reveal a complex neuronal and astrocytic phenotype upon lineage-restricted deletion of Lrp1 gene early during development, with prominent seizures, ventricular volume enlargement, and cortically induced ataxia.

The presence of seizures is a novel characteristic of an Lrp1 ablation mouse line, as other lines only show traits of hyperactivity (Q. Liu et al., 2010; May et al., 2004). The emergence of seizures in our case can be explained through differences in cell populations affected by the KO. Emx1, the promoter guiding the KO to radial glia, is expressed in the frontal brain, but not in the ganglionic eminence. Therefore glutamatergic, but not GABAergic neurons lack Lrp1 in our model (Gorski et al., 2002; Kummer, Kirmse, Witte, & Holthoff, 2012). The development of seizures early in postnatal life, as seen in our study, may in consequence be favored by an imbalance between excitation and inhibition. This is in contrast to SynapsinCre-Lrp1−/− mice where the KO affects both glutamatergic and GABAergic neurons, and the excitation-to-inhibition ratio is thereby reportedly unaltered (Chiappalone et al., 2009; May et al., 2004). In agreement with this interpretation, our hippocampal ex vivo patch-clamp recordings revealed enhanced excitability of hippocampal pyramidal neurons in P28 Emx1Cre-Lrp1−/− animals. Which inhibitory cell types are affected, as well as the regions in which they are affected, in our model, shall be the focus of future studies.

Although the circuitry resulting in ataxia in the current model remains to be elucidated, Emx1-expressing medium spiny neurons can potentially contribute to its development, as they are involved in both movement facilitation and inhibition (Cocas et al., 2009).

A striking phenotype characteristic found in Lrp1 KO mice is the enlargement of the lateral ventricular volume. Dysfunctional aquaporin 4-containing perivascular astrocytic endfeet can offer a possible explanation behind this feature. Aquaporin, as a transmembrane water channel, is especially crucial for extracellular space volume regulation and waste clearance via the glymphatic system (Eidsvaag, Enger, Hansson, Eide, & Nagelhus, 2017; Haj-Yasein et al., 2011). The depletion of aquaporin specifically in astrocytes impairs water influx and efflux from the brain parenchyma (Haj-Yasein et al., 2011).

Although no deficits in cortical thickness, neuronal or hippocampal nuclei numbers were detected in the current study, further research is needed to determine whether neuronal apoptosis also contributes to the lateral ventricular enlargement. A detailed analysis of the hippocampal volume, for example, using light-sheet microscopy, could furthermore help clarify the detected volume differences between the dorsal, intermediate and ventral hippocampi.

The current study demonstrates that Emx1Cre-Lrp1−/− mice display a neuronal phenotype that contributes to seizure generation. The scaffolding protein PSD-95 is found in the postsynapse of excitatory synapses and plays a crucial role in the stability of dendritic spines (Chen et al., 2011; El-Husseini, Schnell, Chetkovich, Nicoll, & Bredt, 2000). Lrp1 KO mice exhibited a significant reduction of PSD-95 expression in P28 and P56 tissue, consistent with findings of May et al. (2004), Liu et al. (2010), and Nakajima et al. (2013). The delayed reduction of PSD-95 levels in the cortex of the Lrp1 KO animals additionally suggests a heterogeneous response of different dorsal telencephalon regions to the deletion of Lrp1. Interestingly, PSD-95 levels are also found downregulated in epileptic patients and animal models of kainate induced epilepsy (Wyneken et al., 2001, 2003), but the mechanisms are not yet fully understood. Altered PSD-95 levels upon Lrp1 loss emerge thereby as a potential contributor to the epileptic phenotype also in humans.

In addition to changes in the postsynaptic compartment upon Lrp1 loss, another Lrp1 mutant indicates that NMDAR surface expression is dependent on Lrp1 NPXY2 motif mutations that are associated with hyperactivity and cognitive deficits (Maier et al., 2013). Our Emx1Cre-Lrp1 mouse model corroborates this, as Lrp1 KO neurons displayed unaltered total NMDAR GluN1 levels but increased GluN1 surface levels. This is traceable to the decreased endocytosis of GluN1-containing NMDARs following Lrp1 loss and the resulting retention of the NMDAR on the neuronal surface (Maier et al., 2013). Considering that the elevation of extrasynaptic NMDARs is suggested to be involved in the pathophysiology of epilepsy (Frasca et al., 2011; Parsons & Raymond, 2014), we propose that NMDAR upregulation in Emx1Cre-Lrp1−/− mice contributes to seizure generation. Whether it is confined to the extrasynapse or not remains an interesting question for future studies.

It is conceivable that the described changes in the neuronal line-age do not suffice to unravel the current complex phenotype, as none of the Lrp1 mutants that target neurons display epilepsy. Given that
the current Lrp1 KO occurs specifically in radial glia and their progeny we therefore turned to studying glial cells in our mutant.

Astrocytes are a highly diverse cell type, forming at least five distinct subpopulations present across different brain regions and developmental stages, that abound with Lrp1 (Auderset et al., 2016; John Lin et al., 2017; Yoon, Walters, Paulsen, & Scarisbrick, 2017). Mirroring their heterogeneity, astrocytes are vital for many processes including synaptogenesis, myelination, synaptic function and establishment of neural circuits. Astrocytic malfunctioning is thereby associated with various neurodegenerative disorders (Clarke &

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**FIGURE 10** P28 Emx1Cre-Lrp1−/− mice show decreased hippocampal Sox9 cell number and increased GFAP and Vimentin. (a–h) Sox9 and Vimentin cells in the dorsal (a–d) and ventral (e–h) hippocampal dentate gyrus of P28 WT and KO mice (MW, b: W = 23, c: W = 24, d: W = 0, f: W = 28, g: W = 36, h: W = 0, N = 3 WT, 3 KO). [l–m] GFAP and Vimentin cell coverage in the granule layer of the dentate gyrus and the hilus (MW, j: W = 0, k: W = 0, l: W = 22, m: W = 0, N = 3 WT, 3 KO). Red bracket in (a) marks the subgranular zone, the area marked by the red dashed line traces the area of the hilus. Red arrowheads in (l) point to stem cell processes. For all, representative images are shown. Scale bars in (a–c) represent 100 μm. Data are expressed as median with interquartile ranges (* indicates p < .05, ** indicates p < .01). A.U., arbitrary units; DG, dentate gyrus; GFAP, glial fibrillary acidic protein; GL, granule layer; KO, knock-out; MW, Mann–Whitney U test; SC, stem cell; Sox9, transcription factor Sox9; Vimentin, type III intermediate filament protein; W, statistics value for the MW test; WT, wildtype; μm, micrometer; %, percent; #, number of. Supporting Information is presented in Figure S10D.
Barres, 2013; Molofsky et al., 2012; Papouin, Dunphy, Tolman, Foley, & Haydon, 2017).

No significant alterations in radial glia cell fiber intensity or astrocyte cell number were observable at E18.5. A more detailed analysis of embryonic tissue is however needed in order to investigate radial glia cell properties in our model more thoroughly. Given that Lrp1 functions can be to some extent compensated by other receptors and proteins, its deletion in vivo may not have the same impact or time course as in vitro. Despite no changes in the astrocyte lineage embryonically, we did detect a mild increase in Sox9-Olig2 double positive cells at the investigated stage. This interesting finding may indicate that the oligodendrocytic lineage is impacted by our in vivo KO as it is upon in vitro Lrp1 deletion in NSPCs (Safina et al., 2016), as well as in NG2-positive glia in vivo (Schafer et al., 2019) and should be therefore studied further.

In postnatal Emx1Cre-Lrp1<sup>−/−</sup> mice, prominent astroglial number and property changes have been observed. A developmental upregulation of GFAP was found starting from P28 in KO cortices and hippocampi. S100b numbers were unaltered and astrocyte proliferation was also not influenced at this stage. Given that GFAP levels in physiological conditions can largely differ not only between various brain regions and developmental stages, but also between neighboring astrocytes, astrocytic reactivity, visualized by a higher density of GFAP positive cells seen in this study is due to the presence of higher GFAP protein levels (Liddelow & Barres, 2017). In line with this, no changes in ALDH1L1 protein levels were detected in P28 mice, a broad, highly specific antigen for astrocytes (Cahoy et al., 2008). Corroborating this interpretation, a previous study by Q. Liu et al. (2010) reported that the elimination of Lrp1 in neurons causes GFAP upregulation, albeit in the absence of seizures (Q. Liu et al., 2010).

Our further analysis confirmed that reactive gliosis is present in our model: not only GFAP but also Vimentin levels were increased in the cortex and reactive stem cell fibers were found in the DG of P28 mice. The combined analysis of Sox9 and GFAP with the proliferation marker Ki67 further revealed that, astrocytes located especially at the level of the piriform cortex, seem to be the first to respond to altered excitability. This is an interesting finding as the piriform cortex, studied mainly in the kindling model of epilepsy, is thought to be vital for seizure initiation, propagation and generalization (reviewed by Cheng, Wang, Chen, & Chen, 2020).

**FIGURE 11** P14 Emx1Cre-Lrp1<sup>−/−</sup> mice show no changes in hippocampal Sox9 and GFAP. (a–h) Sox9 and GFAP cells in the dorsal (a) and ventral (e) hippocampal dentate gyrus of P14 WT and KO mice (MW, b: W = 6, c: W = 12, d: W = 5, f: W = 8, g: W = 13, h: W = 2, N = 3 WT, 3 KO). Red bracket in (a) marks the subgranular zone, the area marked by the red dashed line traces the area of the hilus. For all, representative images are shown. Scale bars in (a and e) represent 100 μm. Data are expressed as median with interquartile ranges. A.U., arbitrary units; DG, dentate gyrus; GFAP, glial fibrillary acidic protein; GL, granule layer; KO, knock-out; MW, Mann-Whitney U test; SC, stem cell; Sox9, transcription factor Sox9; W, statistics value for the MW test; WT, wildtype; μm, micrometer; #, number of. Supporting Information is presented in Figure S10C.
FIGURE 12 Altered glutamate transporter levels in young Emx1Cre-Lrp1−/− mice. (a and b) GLT-1 protein levels in P7 tissue (MW, a: cortex: W = 19, b: hippocampus: W = 14, N = 6 WT, 6 KO, two repetitions for each sample). (c and d) GLT-1 protein levels in P14 tissue (MW, c: cortex: W = 1, d: hippocampus: W = 8, N = 4 WT, 5 KO, three repetitions for each sample). (e and f) GLT-1 protein levels in P56 tissue (MW, e: cortex: W = 17, f: hippocampus: W = 16, N = 7 WT, 6 KO, two repetitions for each sample). (g and h) GLAST protein levels in P7 tissue (MW, g: cortex: W = 8, h: hippocampus: W = 3, N = 6 WT, 6 KO, two repetitions for each sample). (i and j) GLAST protein levels in P14 tissue (MW, i: cortex: W = 6, N = 6 WT, 6 KO, three repetitions for each sample, j: hippocampus: W = 12, N = 6 WT, 5 KO, three repetitions for each sample). (k and l) GLAST protein levels in P56 tissue (MW, k: cortex: W = 11, l: hippocampus: W = 13, N = 7 WT, 6 KO, two repetitions for each sample). Data are expressed as median with interquartile ranges (* indicates p < .05). For all Western blots, representative blots are shown. GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter 1; kDa, kilo Dalton; KO, knock-out; MW, Mann-Whitney U test; N, number of animals; P, postnatal day; W, statistics value for the MW test; WT, wildtype. Supporting Information is presented in Figure S12C.
It has been already established that alterations in the expression and function of astrocytes cause disturbances in neuronal functioning (Steward, Torre, Tomasulo, & Lothman, 1992). However, whether reactive astrocytes are the cause, or rather the consequence of neuronal dysfunction is still debated (Robel & Sontheimer, 2016; Verkhratsky et al., 2012). Supporting the first possibility and in line with our results, a recent study revealed that a conditional deletion of β1-integrin in radial glia cells leads to a chronic reactive astrogliosis,
sufficient to induce spontaneous seizures, without gross brain abnormalities, or pronounced inflammation (Robel et al., 2009, 2015). Interestingly, and analogous to our current in vivo and previous in vitro findings, ablation of β1-integrin from neural stem cells of the hippocampal DG leads to an increase of astrocyte progeny, in conjunction with a decrease of the number of radial neural stem cells (Brooker, Bond, Peng, & Kessler, 2016; Safina et al., 2016). Given Lrp1 is tightly involved in β1-integrin processing (Salicioni, Gaultier, Brownlee, Cheezum, & Gonias, 2004; Wujak et al., 2018), future studies regarding the β1-integrin role in astrocytic maturity and functionality, in the current model, are of value and can help elucidate why Lrp1 loss results in gliosis and how neuronal and astrocytic crosstalk is impacted in epilepsy.

Next to astrocyte reactivity, we detected a transient increase in S100b-positive cell numbers and GLT-1 levels in P14 KO cortices. The increase in P14 S100b-positive cells, but not in Sox9-positive astrocyte numbers, indicate an earlier maturation of cortical astrocytes (Raponi et al., 2007). In line, the increase in GLT-1 levels suggests a possible, tighter NMDAR control in the mutant neonatal cortex (Hanson et al., 2015). Alternatively, as basal c-fos levels were found higher in P14 KO cortices in the current study and given that GLT-1 expression varies depending on seizure occurrence and

**FIGURE 14** Emx1Cre-LRP1−/− mouse derived astrocytes exhibit reduced tPA uptake capabilities. (a) Representative image showing tPA-FITC uptake by WT mouse astrocytes. Marked region in (a) is magnified in inset (a′). (a′) Black arrowheads point to tPA-FITC puncta taken up by WT astrocytes. (b) Representative image showing KO mouse astrocyte tPA-FITC uptake. (c) Quantification of data presented in (a) and (b) (MW, W = 27, N = 5 WT, 6 KO, experiment was repeated three times independently with the same results). (d) Representative image showing tPA-FITC uptake by WT astrocytes after incubation at 4°C. (e) Representative image of albumin-FITC uptake in WT astrocytes after incubation at 37°C. Scale bars in (a, b, d, e) represent 20 μm. Data are expressed as median with interquartile ranges (* indicates p < .05). GFAP, glial fibrillary acidic protein; KO, knock-out; MW, Mann–Whitney U test; N, number of animals; P, postnatal day; tPA, tissue plasminogen activator; W, statistics value for the MW test; WT, wildtype; μm, micrometer.
The intensity of neuronal activity (Hubbard et al., 2016), GLT-1 emerges as an early indicator of circuitry deficits in our model. Together, the transient increase in neonatal hippocampal GLAST and cortical GLT-1 levels suggest biased glutamate transporter density on the cell surface or their altered functionality that favors glutamatergic signaling (Danbolt, 2001; Hanson et al., 2015). In combination with the precocious maturation of astrocytes circuit formation becomes altered, leading to erroneous connectivity between neurons later in life (Robel & Sontheimer, 2016).

Alongside altered glutamate transporter levels, we found differential tPA protein levels in neonatal and juvenile KO mice. In particular, the decrease in tPA levels detected at P7, its increase at P14 and decrease at P28 was found more prominent in the hippocampus than in the cortex. This indicates that tPA levels can be impacted by tPAs' cellular origin and regional environment (Louessard et al., 2016; Stevenson & Lawrence, 2018). As tPA is an immediate-early gene expressed early after seizures (Qian, Gilbert, Colicos, Kandel, & Kuhl, 1993), its transient level changes are also traceable to the varying onset and severity of the seizures in our model.

Upon glutamate application to astrocytes cultured in vitro, tPA is promptly endocytosed via Lrp1 in a clathrin- and dynamin-dependent manner, preventing NMDAR-mediated neurotoxicity (Cassé et al., 2012; Nicole et al., 2001). In our model, cultivated postnatal Emx1Cre-Lrp1−/− astrocytes displayed impaired functionality visualized by reduced tPA uptake capabilities, in line with previous studies (Cassé et al., 2012; Fernandez-Monreal, Lopez-Atalaya, Benchenane, Cacquevel, et al., 2004; Fernandez-Monreal, Lopez-Atalaya, Benchenane, Leveille, et al., 2004). Increasing evidence suggests that astrocyte dysfunction plays a pivotal role in the pathophysiology of epilepsy (Bedner et al., 2015; B. Liu et al., 2017; Robinson & Jackson, 2016). It is appreciated that the disruption of astrocytic tPA uptake leads to an increase in the levels of tPA present in the synaptic

**FIGURE 15** Early astrocytic dysfunction upon loss of Lrp1 in radial glia cells contributes to an impairment of astrocyte and neuronal functioning and the emergence of a severe neurological phenotype. In wildtype mice, the presence of Lrp1 in radial glia cells and their progeny ensures proper maturation and function of astrocytes and neurons that in turn supports stable and adequate glutamatergic signaling. Upon Lrp1 deletion from radial glia cells early in development, the animals exhibit severe alterations in cell signaling that are exemplified by changes in brain morphology, epilepsy and ataxia. Loss of Lrp1 in the astrocytic progeny of radial glia cells disturbs astrocytic maturation, results in GFAP upregulation and impairs astrocytic tPA uptake. Altered astrocytic functionality on top of neuronal deficits: reduced PSD-95 and upregulated NMDAR impacts astrocyte-neuronal interactions and thereby synaptic plasticity and predisposes the animal to seizures. GFAP, glial fibrillary acidic protein; KO, knock-out; Lrp1, low-density lipoprotein receptor-related protein-1; NMDAR, N-methyl-D-aspartate receptor; PSD-95, postsynaptic density protein 95; tPA, tissue plasminogen activator; WT, wildtype.
Emx1Cre-Lrp1

Lrp1 as a new gene of interest at the core of epileptogenesis and the buildup of hyperexcitability, and seizures. Our results highlight morphological changes during mouse brain development that lead to effects on cell signaling in developing Emx1Cre-Lrp1 mice: tPA can potentially remain in the synaptic cleft longer, diffuse further and activate both synaptic and extrasynaptic NMDARs (Bertrand et al., 2015; Nicole et al., 2001; Parcq et al., 2012; Samson et al., 2008). In conclusion, based on our results and available literature that highlights tPAs’ pivotal role in NMDAR dependent Ca²⁺-influx and NMDAR surface diffusion (Lesept et al., 2016), we put forward that tPA imbalance caused by astrocytic deficits contributes to the development of the severe epileptic phenotype in the current mouse line.

To sum up the findings of our study, we propose that Lrp1 deficiency early in development—in both neurons and astrocytes—results in the deterioration of NMDAR-Lrp1-tPA signaling of these cells that profoundly impacts synaptic plasticity and glutamatergic signaling in the developing brain (Figure 15). Our study provides evidence that early astrocytic dysfunction is an important factor contributing to morphological changes during mouse brain development that lead to the buildup of hyperexcitability, and seizures. Our results highlight Lrp1 as a new gene of interest at the core of epileptogenesis and the Emx1Cre-Lrp1−/− mouse as an ideal tool to study the significance of both astrocytic and neuronal malfunctioning on proper circuit establishment.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data is available upon reasonable request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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