Synaptic Phospholipid Signaling Modulates Axon Outgrowth via Glutamate-dependent Ca\textsuperscript{2+}-mediated Molecular Pathways

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Abstract

Altered synaptic bioactive lipid signaling has been recently shown to augment neuronal excitation in the hippocampus of adult animals by activation of presynaptic LPA2-receptors leading to increased presynaptic glutamate release. Here, we show that this results in higher postsynaptic Ca\textsuperscript{2+} levels and in premature onset of spontaneous neuronal activity in the developing entorhinal cortex. Interestingly, increased synchronized neuronal activity led to reduced axon growth velocity of entorhinal neurons which project via the perforant path to the hippocampus. This was due to Ca\textsuperscript{2+}-dependent molecular signaling to the axon affecting stabilization of the actin cytoskeleton. The spontaneous activity affected the entire entorhinal cortical network and thus led to reduced overall axon fiber numbers in the mature perforant path that is known to be important for specific memory functions. Our data show that precise regulation of early cortical activity by bioactive lipids is of critical importance for proper circuit formation.

Key words: axon outgrowth, bioactive phospholipids, Ca\textsuperscript{2+}-signaling, early synchronized activity, entorhinal–hippocampal formation
Introduction

The entorhinal–hippocampal projection (Amaral and Witter 1989) has gained special attention due to its importance for memory formation and spatial navigation (Moser et al. 2008). Formation of this pathway follows a precise spatiotemporal pattern of axon outgrowth (Del Rio et al. 1997). In the developing entorhinal cortex (EC), periodic spontaneous glutamate receptor-mediated events parallel the outgrowth of the entorhinal–hippocampal projection and show a characteristic time-locked increase during the first postnatal days (Sheroziya et al. 2009; Unichenko et al. 2015). In the early postnatal animal, space-encoding neurons show characteristic activity when the animal first starts to explore its environment, suggesting that the EC forms functional local excitatory connections to the hippocampus already during the early postnatal period (Langston et al. 2010). A role for early excitatory local activity in axon growth has been described in subcortical structures (Mire et al. 2012), indicating that early neuronal activity in the EC may be essential for the development of proper adult connectivity.

Glutamate not only acts as a neurotransmitter at excitatory synapses in the entorhinal–hippocampal system but also regulates axon growth (Yamada et al. 2008). Application of glutamate to the somatodendritic area induced axon retraction via calcium waves that propagated from the cell body to the growth cone (Yamada et al. 2008). This is in line with recent data showing that electrical activity induced by optogenetic stimulation decreased horizontal axon growth of cortical neurons (Malyshkevaya et al. 2013). Here, we show that glutamatergic signaling in the EC depending on proper regulation by synaptic bioactive phospholipid signaling critically influences axonal outgrowth and circuit formation via Ca$^{2+}$-dependent pathways. This signaling pathway is regulated by the postsynaptic molecular plasticity related gene 1 (PRG-1/PLPR4), which is an integral membrane protein and has a homology to lipid phosphate phosphatases, and is mediated via the presynaptic LPA$_2$-receptor (R) influencing glutamate release (Trimbach et al. 2009). PRG-1 expression starts around birth (Brauer et al. 2003), concomitant with the development of the entorhinal–hippocampal perforant path (Del Rio et al. 1997), and increases towards the end of the second postnatal week (see Fig. 5a–c). This expression pattern suggests that entorhinal–hippocampal projection would be affected by increased glutamatergic transmission caused by PRG-1 deficiency. Indeed, in PRG-1$^{-/-}$ mice, which lack the postsynaptic PRG-1-dependent regulation of phospholipid signaling, we detected a premature appearance of early spontaneous glutamatergic activity in the EC by 2 days. This was mediated by presynaptic LPA$_2$-Rs and led to Ca$^{2+}$-dependent molecular signaling to the axon growth cone resulting in reduced axon growth velocity of entorhinal fibers. Reduced axon growth speed was due to specific alterations in Ca$^{2+}$-dependent signal transduction (Wayman et al. 2008) including increased phosphorylation of CaMKI but not of other members of the Ca$^{2+}$/Calmodulin(CaM)-pathway. A target of the Ca$^{2+}$/CaM-dependent protein kinases (Takeamura et al. 2009; Saito et al. 2013), LimK1, which inactivates coflin activity via phosphorylation (Mizuno 2013), showed reduced phosphorylation levels in the EC. Since coflin negatively regulates actin dynamics and destabilizes axons via actin depolymerization (Mizuno 2013; Saito et al. 2013), reduced coflin phosphorylation levels explain the negative effect on axon outgrowth of entorhinal fibers to the hippocampus. Our findings show that precise regulation of local excitatory networks is critical for circuit formation in the entorhinal–hippocampal connection mediated via Ca$^{2+}$-dependent axonal signaling pathways.

Materials and Methods

Transgenic Animals and Breeding

PRG-1$^{-/-}$ and PRG-1$^{-/-}$/LPA$_2$-R$^{-/-}$ mice and their littermate controls were obtained as described (Trimbach et al. 2009). For coculture experiments, Thy-1.2-EGFP_L17 (Caroni 1997) and C57BL/6 TgN(Bact-EGFP) Osb 1 (Okabe et al. 1997) mice were crossbred with PRG-1$^{-/-}$ mice. All experiments involving animals were conducted in accordance with national laws for the use of animals in research and approved by the local ethical committee.

Ca$^{2+}$-Imaging and Electrophysiology In Vitro at P5

Slice preparation, extracellular field potential recordings, Ca$^{2+}$-imaging and single cell electrophysiology were performed according to standard procedures (Unichenko et al. 2015, 2016). EEPCs were elicited by focal electrical stimulation through a glass pipette filled with the ACSF (~10 MΩ). The stimulation pipette was always positioned within layer 2 of MEC. N-(2,6-dimethylphenylcarbamoylmethyl)-triethylammonium bromide (QX 314, 2 mM) was added to the intracellular solution to prevent generation of action potentials in the tested neurons. An isolated stimulation unit was used to generate rectangular electrical pulses. Pulse duration was set to 0.2 ms. Pulse intensity was adjusted to activate a unitary synaptic input (minimal stimulation). Stimulation was accepted as minimal if the following criteria were satisfied: 1) eEPSC latency remained stable (<20% fluctuations), 2) lowering stimulus intensity by 20% resulted in a complete failure of eIPSCs, and 3) an increase in stimulus intensity by 20% changed neither mean eEPSC amplitude nor eEPSC shape. In addition, eEPSCs were inspected at a holding potential of ~50 mV, Nernst Cl$^{-}$ reversal potential. Depolarizing eEPSCs recorded at this membrane potential confirmed the glutamatergic nature of the evoked signals. Typical pulse intensity was between 2 and 4 µA.

Surgical Preparation and In Vivo Recordings in P5 Mice

Briefly, after 30–60 min recovery from the surgical procedure (Yang et al. 2013; Reyes-Puerta et al. 2015), a 4-shank 16-channel electrode (125 µm horizontal shank distance and 50 µm vertical inter-electrode distance, 1–2 MΩ; NeuroNexus Technologies) or an 8-shank 128-channel electrode (200 µm horizontal shank distance and 75 µm vertical inter-electrode distance, 1–2 MΩ; NeuroNexus Technologies) was inserted into cortical layer II/III of the anesthetized mouse to obtain FP recordings. One hour after the electrode was inserted into the EC, spontaneous activity was recorded for 1 h. Usually, spontaneous events were recorded from 1 to 2 groups of channels. The channel with maximum occurrence of spontaneous events was chosen for further analysis.

Data Analysis of In Vivo Recordings

FP data were imported and analyzed offline using MATLAB software version 7.7 (MathWorks). In each experiment, 900 s spontaneous extracellular local FP recording was used for the analysis. To detect spontaneous events, raw data were filtered between 1 and 80 Hz using a Butterworth 3-order filter. Spontaneous events were detected as FP deflections exceeding 5 times the baseline standard deviation (SD). The events were analyzed in their occurrence,
duration, and frequency with maximal fast Fourier transform
ation power within event. MUA was detected in 200 Hz highpass
filtered signals by applying a threshold at 7 times the baseline
SD. We calculate the MUA firing rate in channels located in MEC
and further calculate the mean MUA firing rate from the chan-
nels with MUA firing rate >0.

Measurement of Axon Outgrowth Using Ex Vivo
Organotypical Slice Cultures

Live-Imaging Measurements
Organotypic slice cultures containing EC from GFP-expressing
transgenic mice and hippocampus from non-GFP transgenic
mice were performed as described. After DIV3 cocultures were
placed in a preheated chamber (37°C) aerated with humidified
5% CO₂ ambient air (Ludin chamber, Life Imaging Services).
Imaging of outgrowing entorhinal axons in living slice cultures
was performed using a microscope (TCS SP2, Leica) equipped
with a 2-photon laser (Coherent, Titan: Saphire Chameleon).

GFP fluorescence was obtained at an excitation wavelength
of 900 nm and was imaged using a 20x water objective (Olympus
XLMPlanFL 0.95 NA). Axon growth velocity was calculated
measuring the distance covered by single axons over time.
Before imaging, slices were equilibrated for at least 1 h in the
imaging chamber. Multiple optical sections (up to 30 sections,
z-spacing of 0.8 μm) were collected every 10 min for up to 3 h.
This observation condition did not induce photobleaching of
GFP fluorescence nor did it alter axon outgrowth speed during
the experiment. According to experimental conditions, 0.5 mM
glutamate was directly applied to the medium. For data ana-
lysis, optical sections were reconstructed into single projection
images for each time point using the maximum brightness
operation of the Leica software. Each data point represents the
axon outgrowth speed of 1 axon.

Figure 1. Altered phospholipid signaling regulating basal glutamatergic transmission increased neuronal firing probability in individual neurons. (a) Original traces showing mEPSCs from layer II/III pyramidal neurons voltage clamped at −70 mV. (b) Neurons from PRG-1−−/− mice (n = 9 neurons) demonstrated more mEPSCs compared with those from their WT litters (n = 11 neurons), while average amplitude and cumulative mEPSC amplitude distribution of these events was similar between the 2 groups (Mann-Whitney test). (c) Representative averaged eEPSCs (20 trials in both cases) recorded from layer 2 MEC neurons in slices from P5 WT (black) and P5 PRG-1−−/− (red) mice. (d) Dependence of the paired pulse ratio (PPR) on the interstimulus interval in slices from WT (black) and PRG-1−−/− (red) animals. (e) Significantly larger eEPSC amplitudes (left panel) and smaller failure rates (right panel) were observed in slices from PRG-1−−/− mice as compared with slices from WT littermates (n = 8 WT and 8 PRG-1−−/− neurons, unpaired Student’s t-test). (f) Quantitative analysis of active cell numbers identified by Ca²⁺-imaging revealed significantly increased numbers of spontaneously active cells in layer II/III of the MEC in PRG-1−−/− slices at P3 and P5 (P3, n = 27 WT and 30 PRG-1−−/− slices, Mann-Whitney test; P5, n = 33 WT and 25 PRG-1−−/− slices and P7, n = 23 WT and 27 PRG-1−−/− slices, t-test). All data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 2. Increased early neuronal activity inhibits axon growth. (a) Organotypic cocultures of a GFP-expressing EC and a nonfluorescent hippocampus allow for the assessment of single fiber growth speed. See also Fig. S2a,b. (b) Analysis of WT axon growth speed after 3 days in vitro cultivation (DIV3, n = 41 axons) and DIV5 (n = 18 axons). (c1) Slower axon growth speed at DIV5 correlated with a shift from a fast advancing category (adv, Mann–Whitney, c2) to a slow outgrowth category (slow, Mann–Whitney, c3). See also Fig. S2c. (d) Alignment of representative outgrowing fibers over 60 min. Note the decrease in outgrowth speed of DIV5 WT and of DIV3 PRG-1−/− axons. (e) Fiber growth speed of DIV3 axons originating from WT and from PRG-1−/− EC slices into a PRG-1−/− hippocampus (n = 15 WT axons and 12 PRG-1−/− axons) or into a WT hippocampus (n = 41 WT axons and 15 PRG-1−/− axons) assessed in live-imaging experiments. Remarkably, the outgrowth speed of axons originating from PRG-1−/− EC slices was significantly lower independent of the PRG-1 expression in the target region. (f1) Axon growth speed categories of DIV3 WT and PRG-1−/− axons in % of the live-imaging time. (f2) The percentage of time PRG-1−/− axons displayed a fast advancing outgrowth was significantly decreased when compared with WT axons. (f3) In line, the periods PRG-1−/− axons were found in a slow outgrowth category significantly increased. See also Fig. S2d. (g1) Live imaging of fiber growth before and after application of 500 µM glutamate. Arrows indicate the starting point of growing axons. (g2) Metric display of fiber growth of the depicted axons over 140 min (70 min control (white), 70 min glutamate application (red)). (h) Analysis of fiber growth speed (n = 14 WT axons). (i) Fiber growth speed at the beginning and the end of glutamate application (n = 11 WT axons). All data represent mean ± SEM. For statistical analyses, parametric data were calculated using an unpaired t-test or a paired t-test when appropriate. *P < 0.05; **P < 0.01; ***P < 0.001.
speed <50% of the mean speed in the fast advance category was attributed to the slow outgrowth category (category 2). The time when axon growth speed was below 10% of the mean speed in the fast advance category down to 0 µm/h was defined as a pause of axon growth (axon stop category 3). Negative axon growth was regarded as retraction (category 4).

**Measurement of Axonal Outgrowth in Collagen-Embedded Slice Cultures**

Axon outgrowth assays were performed and analyzed as described (Holtje et al. 2009) using ImageJ. Briefly, EC slices were prepared at postnatal day 0/1 and were embedded in a
collagen I matrix on glass slides and covered with nutrition medium. An inhibitor cocktail containing TTX (1 μM), CNQX (20 μM), and nifedipine (10 μM) (all from Sigma) was added to the cultures that were incubated for 48 h before microscopic analysis. To evaluate the axonal outgrowth of the explants, neurite density was analyzed 100 μm in front of the concave side of the explant with ImageJ. Each data point represents measurements in 1 entorhinal slice culture. Axon outgrowth was normalized to the mean value of the corresponding PRG-1-expressing slices.

Measurement of Axonal Ingrowth of GFP-expressing Axons into the Dentate Gyrus

Experiments were performed according to established protocols (Walsh et al. 2015a; Vogt et al. 2012). Briefly, after 3 days in vitro, (DIV3) or after DIV7, respectively, cultures were fixed with 4% paraformaldehyde (PFA), resliced, coverslipped, and imaged with a fluorescence microscope (Olympus, BX 50) equipped with a Cool SNAP ES digital camera (Roper Scientific). To measure axonal ingrowth, fluorescence intensity of the GFP-positive EC projection was determined in the ML using the software ImageJ or MetaMorph (Molecular Devices). Two regions of interest (ROIs, all ROIs were identical) comprising the full width of the ML were randomly selected in the ML and in the DG. The average fluorescence in the DG was used to determine the background fluorescence, and the difference in fluorescence intensity between the ML and the DG reflected the specific fiber ingrowth in the ML. Data were normalized to the fluorescence intensity of fiber ingrowth of WT→WT (Fig. 3f/g).

Immunohistochemistry

Cocultures with entorhinal cortices from Thy-1.2-EGFP.L17 mice growing into the PRG-1-deficient hippocampal target tissue (EGFP-negative) were fixed with 4% PFA, resliced, and incubated with an antibody against Calbindin (Swant, Bellinzona) or with acustom-made antibody against PRG-1 (Tribuch et al. 2009). For developmental expression studies, antibodies against autotaxin (Tanaka et al. 2004), LA1-receptor (Tribuch et al. 2009), VGluT1 (Synaptic Systems), GFAP (DAKO), β-Gal (Abcam), and Calbindin and Calretinin (Swant) were used. Secondary antibodies Al 488 or Al 568 (Invitrogen) were applied overnight at 4°C. For DAB conversion, biotinylated secondary antibodies were used and DAB-staining was performed as described (Vogt et al. 2012). For assessment of PRG-1 expression, heterozygous PRG-1-deficient mice expressing a β-Gal reporter were used (for detailed description, see Tribuch et al., 2009). Confocal imaging was performed on a Leica TCS SP8 or on a Leica TCS SL confocal laser scanning microscope.

Analysis of Phosphorylation Levels

Western blot analysis of brain lysates or of purified phosphoproteins was performed following standard procedures. Briefly, P5 animals were killed and entorhinal cortices from 2 animals (for WT versus PRG-1/−) or 3 animals (for WT versus LPA2-Δ− or PRG-1/−/LPA2-Δ−/−, respectively) were pooled to one biological sample and analyzed as described in the figure legends for the respective experiment. A total of 2.5 mg of protein was then loaded onto the PhosphoProtein purification column (Qiagen). Fractions containing phosphorylated proteins were reduced to 200 μL using Startorius stedim biotech Columns. Protein levels using western blot analysis was performed using following antibodies: CamKI (1:2000; AbCam), Calmodulin (1:1000; Millipore), CamKKK (1:500; AbCam), CamKIV (1:1000; Cell Signaling), pCamKI (1:100; provided by Naohito Nozaki and described in Tokumitsu et al., 2004)), LimK1, pLimK1/2, Cofilin and pCofilin (1:1000; Cell Signaling), and beta-actin (1:10000; MP Biomedicals, LLC). Subsequently, blots were processed for 1 h at room temperature with HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (1:5000; Dianova). Densitometric analyses were performed using ImageJ.

Viral Infection and Fiber Outgrowth Assessment

Complex hippocampal-entorhinal slice cultures were prepared from 18 pregnant conditional PRG-1−/− mice and infected using an AAV expressing mCherry or Cre and mCherry under the synapsin promoter. In the AAVs, a 480 bp fragment of the synapsin (Syn) promoter (Kugler et al. 2003) controlled neuronal expression of Cre-mCherry, whereby a 2A-mediated peptide cleavage linker (Donnelly et al. 2001) was introduced between Cre recombinase and mCherry to enable the production of 2 separate protein products. The production of the AAV1/2 EGFP (titer 1.0 × 1013 copies/ml) of the AAV6 Syn-mCherry (titer 1.05 ×1010 copies/ml) and of the AAV6 Syn-Cre-mCherry (titer 6.55 × 1013 copies/ml) was performed as previously described (Guggenhuber et al. 2010). After slice preparation, Syn-mCherry, Syn-Cre-mCherry or EGFP expressing AAV were injected at a dilution of 1:5 in the EC using glass pipettes attached to a microinjection device (Toohey Spritzer). In line with recent data (Aschauer et al. 2013), we did not see differences in infection efficiency between these 2 AAV serotypes. After 5 days, in vitro (DIV5) slices were fixed, resliced, and restained for a better visualization of mCherry filled axons (1:1000; Rockland). Fluorescent images were taken on a Leica SP8 equipped with a white light laser and HyD detectors for visualization of the axonal projection. 7.5 μm thick image stacks were used in the maximal projection. Image analysis was performed with ImageJ. To avoid bias by unspecified background staining, a threshold was measured in areas devoid of axonal projections (e.g., granule cell layer of the DG, which contains the cell bodies) and subtracted from the images. Gray values were measured in the ROIs that comprised the entorhinal performant path projection in the ML of the DG or the infected neurons in the EC. Since AAV application may infect varying amounts of EC neurons, gray values of the EC performant path projection was correlated to the number of infected cells and calculated as a ratio to the gray values of the infected EC-cells. Analysis of PRG-1 expression upon cre-infection was performed by immunofluorescent staining using a custom-made rabbit anti-PRG-1 antibody as described (Tribuch et al. 2009).

Stereological Analysis of Entorhinal Perforant Path Axons

Myelin staining was performed according to standard procedures and was quantified using unbiased high-precision stereological methods (Vogt et al. 2009). Briefly, brains were cut in serial sections on a vibratome, and every forth section was selected and stained. Definition of the ROI in the hippocampus: EC fibers crossing the hippocampal fissure were stereologically counted. This ROI was delimited by characteristic anatomical formation of the hippocampus and the fimbria. Every second section of the following 10 was used for stereological assessment. The number of EC fibers in individual animals was...
normalized to the mean of the WT group or to the mean of the LPA2-R−/− group representing the strain background.

Statistical Analysis
After assessing for normal distribution (using the Kolmogorov–Smirnov test), statistical analyses between the 2 groups were calculated as using a t-test for normal distributed data or with a Mann–Whitney U test for nonparametric data. Statistical analyses between more than 2 groups were performed using an one-way analysis of variance with a Bonferroni correction for nonparametric data. All statistical calculations were performed with GraphPad Software (GraphPad Software, Inc.).

Results
Appearance of Excitatory Activity and Postsynaptic Ca2+ Transients in Developing Entorhinal Neurons Depends on Phospholipid Signaling
Periodic spontaneous glutamatergic events in the EC incrementally develop during the first postnatal week (Unichenko et al. 2015) and decrease towards the end of the second postnatal week (Sheroziya et al. 2009). We investigated the role of bioactive phospholipids in regulation of these local excitatory activity and their consequences for circuit formation. To this end, we used PRG-1−/− mice which, at adult ages, were reported to have an increased synaptic lysophospholipid signaling resulting in enhanced presynaptic glutamate release mediated via presynaptic LPA2-R (Trimbuch et al. 2009).

First, we analyzed neuronal activity at single cell level at P5 in layer II of the EC. PRG-1−/− neurons displayed significant higher mEPSC frequencies with unaltered amplitudes (Fig. 1a,b, Table S1). Since altered mEPSC frequency implies a presynaptic effect, we measured paired pulse ratios to prove for this assumption. Therefore, we recorded evoked EPSC (eEPSCs) elicited by paired pulse electrical stimulation in the presence of GABA_A receptor blocker gabazine (10 μM, Fig. 1c). Paired pulse ratio was significantly higher in wild-type (WT) mice as compared with PRG-1−/− littermates (Fig. 1c,d). In PRG-1−/−, the mean amplitude of eEPSCs was increased, while failure rate, i.e., number of trials in which an electrical pulse failed to elicit the first eEPSC in a pair, was decreased (Fig. 1e). These findings reflect an increased basal presynaptic glutamate release and neuronal hyperexcitability of PRG-1−/− developing entorhinal neurons as previously shown for CA1-neurons in adult PRG-1−/− animals (Trimbuch et al. 2009). We next analyzed which type of glutamate receptor mediates the effects of the increased presynaptic release using subtype-specific inhibitors. As the mean amplitude of mEPSCs is small, we recorded eEPSCs at P5, elicited in MEC L2 neurons by electrical stimulation in close proximity to a cell of interest in the presence of gabazine (10 μM), a GABAA receptor blocker. NBQX (10 μM), a selective AMPA (but not kainate) receptor antagonist, completely blocked eEPSCs both in slices from PRG-1−/− animals (n = 4), and from their WT littermates (n = 5, data not shown). However, NASPM (25 μM), a Ca2+-permeable AMPA receptor blocker failed to affect eEPSCs both in slices from PRG-1−/− animals or from their WT littermates (Fig. S1d,e), suggesting that mostly Ca2+-impermeable AMPA receptors mediate glutamatergic synaptic transmission. UBP302 (10 μM), a selective blocker of kainate receptors, did not influence eEPSC parameters in

Figure 4. Excitatory neuronal activity mediated via bioactive lipid signaling and involving LPA2-Rs controls axon outgrowth of the entorhinal projection. (a) Axon outgrowth number was lower in PRG-1−/− EC explants (when compared with WT EC explants) and rescued to WT levels upon inhibition of neuronal activity (1 μM TTX, 10 μM Nifedipine, 20 μM CNOQ). (b) Analysis of axon outgrowth assays revealed complete rescue of PRG-1-deficient explants to WT levels upon electrical activity blockade (n = 73 WT, 93 PRG-1−/− and 37 PRG-1−/−activity explants; one-way analysis of variance with Bonferroni correction). (c and d) Axon outgrowth number was similar in EC explants from PRG-1-expressing and PRG-1-deficient animals raised on an LPA2-R−/− background (n = 106 LPA2-R−/− and 95 PRG-1−/−/LPA2-R−/− explants; t-test). For better comparison, values in b and d were normalized to the mean of the PRG-1 expressing explants (to WT or to LPA2-R explants, respectively). All data represent mean ± SEM. ***p < 0.001.
PRG-1−/− animals or in WT littersmates (Fig. S1f,g). As both substances did not affect PPR, the presynaptic expression of functional kainate and/or Ca2+-permeable AMPA receptors appears to be unlikely. From these data, we conclude that contributions of both kainate and Ca2+-permeable AMPA receptors to glutamatergic transmission in L2 of mEC at P5 is minor and that postsynaptic responses are predominantly mediated by Ca2+-impermeable AMPA receptors.

Analyses of active cells in layer II of PRG-1−/− MEC slices using Ca2+ imaging revealed significantly increased percentage of active cells exhibiting Ca2+ transients at P3 and at P5 reflecting increased discharge rates in the MEC layer II neurons (Fig. 1f). This shows that immature entorhinal neuron activity is regulated by bioactive phospholipids. Altered phospholipid signaling results in higher basal glutamate release that augments the neuronal firing probability leading to increased Ca2+-concentrations in individual neurons.

**Premature Onset of Excitatory Activity in Entorhinal Neurons Alters Axon Outgrowth**

Neuronal activity was shown to affect axon outgrowth in different systems (Gomez and Spitzer 1999; Mire et al. 2012). We therefore established 2-photon live-imaging in organotypic entorhinal–hippocampal cocultures (Fig. 2a, S Movie1), which maintain all elements of the trisynaptic hippocampal circuitry and are regarded as an ex vivo model displaying a comparable development of the entorhinal projection to the situation in vivo (Frotscher et al. 2000). Organotypic slice cultures were prepared from newborn mice, when entorhinal axons start to enter the dentate gyrus (Del Rio et al. 1997).

Ingrowing axons were imaged after 3 days in vitro (DIV3, which is comparable with P3 in vivo (see also Fig. S2a,b), when excitatory activity of entorhinal neurons was almost not present, and at DIV5 (comparable with P5 in vivo), when robust excitatory discharge activity was observed in WT animals. Axon growth velocity at DIV5 (when entorhinal axons started to contact their targets, the granule cell dendrites (Frotscher et al. 2000)) was significantly decreased when compared with DIV3 (Fig. 2b). This decrease of axon growth speed at arrival in its synaptic target region is in line with axon behavior described in other systems (Stamatakou et al. 2015). Thus, decreased axon growth speed at DIV5 correlates with increased Ca2+ transients in WT slices at P5 (Fig. 3d). In order to understand the mechanisms underlying these developmental changes, we performed detailed analyses of axon growth. Since axon growth is not uniform but rather complex containing periods 1, where axons display a fast outgrowth, 2, where axon growth speed slows down or 3, where axons stop growing or 4, even retract, we have analyzed axon outgrowth according to these categories. Detailed analysis of axon outgrowth dynamics revealed that increased discharge activity of entorhinal neurons at DIV5 (when compared with DIV3) significantly altered pattern switching axon growth from a fast advancing category (adv) to a slow outgrowth category (slow) (Fig. 2c–l). Here, axon growth speed was more than 50% slower than in the fast advancing category (Fig. S2c). Interestingly, DIV5 axons displayed a significant decrease in axon outgrowth speed in the fast advancing category, while the axon growth speed in the slow category was not changed pointing to the fact that increased synchronized neuronal activity specifically affected periods of fast axon outgrowth (Fig. S2c).
To assess the impact of premature onset of excitatory activity of entorhinal neurons on their axon growth in PRG-1<sup>−/−</sup> mice, we analyzed axon outgrowth in slices of PRG-1<sup>−/−</sup> mice at DIV3 and found growth rates that were 25% below the rates of axons derived from WT slices of the same age (Fig. 2d,e). Interestingly, growth rates of PRG-1<sup>−/−</sup> axons were significantly lower independent whether the target region, here the hippocampus, was derived from PRG-1 expressing or from PRG-1 deficient mice (Fig. 2e). These findings argue against a change in attractive properties of the target region in PRG-1<sup>−/−</sup> mice as an underlying cause for the observed changes. To exclude possible bias by transient PRG-1 expression in the growth cone, which might account for a decreased outgrowth speed of axons derived from PRG-1<sup>−/−</sup> neurons, we analyzed WT growth cones in detail but found no PRG-1 expression in these structures (Fig. S2e–h). Moreover, analysis of growth categories revealed that DIV3 axons derived from PRG-1<sup>−/−</sup> entorhinal neurons displayed a similar switch from a fast advancing category towards a slow outgrowth category as observed for DIV5 WT-fibers (Fig. 2f, g). This suggests a specific role of increased discharge activity as cause for decreased outgrowth of axons originating from PRG-1<sup>−/−</sup> entorhinal neurons. In line, outgrowth speed of axons originating from PRG-1<sup>−/−</sup> neurons was decreased in the fast advancing category, while no changes in outgrowth speed were observed in the slow outgrowth category pointing to a specific effect of neuronal activity and postsynaptic Ca<sup>2+</sup> increase on fast outgrowing axons (Fig. S2d). Moreover, developmental analysis of the regions where the perforant path originates (the EC) as well as of the region where it terminates (the EC) did not reveal differences in maturation arguing against bias by an altered maturation of these regions in PRG-1<sup>−/−</sup> animals (Fig. S2i–j). In sum, our data strongly support the idea that altered synaptic lipid signaling—resulting in an increased

**Figure 6.** Premature synchronized early neuronal activity affects axon outgrowth related downstream Ca<sup>2+</sup>-signaling. (a) Region specific analysis of the EC at PS revealed an unaltered total amount of CaMKI (n = 5 samples, each sample containing entorhinal cortices from 2 animals). (b) Phosphoprotein purification revealed a significant increase of CaMKI in the EC of PRG-1<sup>−/−</sup> animals (n = 6 samples as described above). (c) Application of a specific antibody against phosphorylated Thr177 confirmed higher amount of phosphorylated CaMKI in the EC of PRG-1<sup>−/−</sup> animals (n = 7 samples as described above, see also Fig. S3e). (d) In line with normalized neuronal activity and rescued axonal outgrowth, PRG-1 deletion on an LPA2<sup>R−/−</sup> background did not influence the amount of phosphorylated CaMKI (n = 5 samples, each sample containing entorhinal cortices from 3 animals for each genotype). (e and f) Analysis of downstream targets directly modulating actin cytoskeleton polymerization revealed normal LimK1 protein levels (n = 6 WT and 4 PRG-1<sup>−/−</sup> samples) but reduced presence of phosphorylated LimK1/2 (n = 10 WT and 7 PRG-1<sup>−/−</sup> samples). (g and h) In line, total cofilin levels were not altered (n = 5 WT and PRG-1<sup>−/−</sup> samples), while phosphorylated cofilin levels (n = 7 WT and PRG-1<sup>−/−</sup> samples) were decreased. Error bars represent SEM. For comparison of the 2 groups, normal distributed data were analyzed using a t-test; nonparametric data were analyzed using a Mann–Whitney test. Comparisons of more than 2 groups (Fig. 6d) were performed using a one-way analysis of variance with Bonferroni correction. *P < 0.05; **P < 0.01. See also Figure S4.
glutamatergic transmission leading to augmented Ca\(^{2+}\) levels in the corresponding entorhinal parent neurons—decreased axon outgrowth speed of the entorhinal projection towards the dentate gyrus, its target region.

Since increased discharge activity indicated elevated glutamatergic levels in the MEC of PRG-1\(^{-/-}\), we tested this assumption via direct manipulation of axon growth by glutamate application (500 µM). Already 10 min after glutamate application, axon growth rates significantly decreased (Fig. 2g, h) and remained at low levels throughout the whole experiment (Fig. 2h, i). Axon growth velocity was not significantly different after 10 and 70 min of glutamate application arguing against a progressive neuronal damage induced by glutamate (Fig. 2i).

**Premature Onset of Excitatory Activity in Assemblies of Entorhinal Neurons Alters Outgrowth in the Entorhinal–Hippocampal Projection**

An increase in mEPSCs leading to neuronal hyperexcitability may affect large group of neurons eventually leading to epileptic discharges (Fang 2016). Therefore, we next analyzed electrical activity of neuronal assemblies in layer II of the medial EC (MEC) and performed field potential (FP) measurements.
Excitatory Activity Mediated by Bioactive Lipid Signaling Controls Axon Outgrowth in the Entorhinal–Hippocampal System

The effect of excitatory discharge activity was further tested on outgrowing axons from EC explants. Quantitative analysis revealed that axon numbers derived from PRG-1−/− EC slices explanted at P1 and cultivated for 48 h (corresponding to P3 in vivo, a time point when discharge activity was almost absent in WT but significantly increased in PRG-1−/− entorhinal slices) was significantly lower but was rescued to WT levels when neuronal activity was blocked by a cocktail containing TTX, CNQX, and nifedipine (Fig. 4a,b). Since PRG-1 deficiency altered synaptic LPA signaling and application of LPA on WT slices mimicked increased glutamatergic levels present in PRG-1−/− mice (Trimbuuch et al. 2009), we investigated whether reduced outgrowth of axons from PRG-1−/− mice was mediated via presynaptic LPA2− receptors. Using WT and PRG-1−/− mice on an LPA2−/− background that blocks lipid signaling at the presynapse (Trimbuuch et al. 2009), we found no more differences between axon outgrowth from WT and from PRG-1−/− slices (Fig. 4c,d). These results confirm that the PRG-1 effect on axon outgrowth was indeed mediated by presynaptic LPA2− receptors, which is supported by electrophysiological data showing that LPA2− receptors mediate premature onset of neuronal activity upon increased lipid signaling in PRG-1−/− mice (Fig. 3d).

Cell-type-specific PRG-1 Deletion Reduces EC Axon Outgrowth

We next aimed to provide direct evidence for the role of postsynaptic PRG-1 in axon outgrowth from the EC to the hippocampus. Therefore, we performed cell-type-specific PRG-1 deletion using adeno associated virus (AAV) cre-expression in a subset of entorhinal neurons carrying a floxed PRG-1 gene. The used AAVs (serotype 1/2 or 6) have a similar neuronal infection efficiency in the hippocampus (Aschauer et al. 2013). Cre-expression occurred under the synapsin promoter, thereby limiting cre-expression and PRG-1 deletion to infected neurons. Infected neurons were visualized via enhanced green fluorescent protein (EGFP) or mCherry expression, both well-described fluorescent proteins for axonal studies (Bochorishvili et al. 2014; Liu et al. 2014). These fluorescent proteins were co-expressed by the AAVs and stained cell bodies as well as their axons. Control AAVs expressed EGFP only. As shown in Figure 5a, infected EC neurons in control slices (neuronal cell bodies in control slices are shown in Fig. 5d) formed a well-defined axonal projection terminating in the molecular layer (ML) of the DG. However, albeit robust neuronal infection by the cre-expressing virus, the perforant path projection originating from the infected and PRG-1-deficient neurons (shown in Fig. 5e) was significantly weaker (Fig. 5b,c). To prove for efficiency of PRG-1 deletion, we resliced and immunostained cre-transfected slices for PRG-1. As shown exemplarily in Figure 5f, infected EC neurons identified by mCherry expression were clearly PRG-1 depleted, while unaffected neurons located close to infected cells displayed strong PRG-1 expression. These data argue for a critical role of postsynaptic PRG-1 in controlling synaptic phospholipid signaling, excitatory activity, and Ca2+ levels, thereby eventually determining the number of axons that reach their target at a given time during development.
Premature Excitatory Entorhinal Activity Alters Downstream Ca2+-Signaling

Higher presynaptic glutamate release induces higher Ca2+-concentrations in the postsynaptic neuron driving Ca2+-dependent signal transduction (Wayman et al. 2008). However, little is known about the molecular mechanisms induced by higher postsynaptic Ca2+-transients eventually decreasing axon outgrowth. Activity-dependent protein phosphorylation is a key process in regulation and fine-tuning of intracellular functions. We therefore analyzed phosphorylation of downstream molecules of the Calsodulin (CaM)-pathway in the EC of P5 WT and PRG-1−/− animals by phosphoprotein purification and western blotting. Here, we found significantly higher phosphorylation levels of CaMKI, a molecule critically involved in axon outgrowth (Ageta-Ishihara et al. 2009), in PRG-1−/− animals when compared with WT litters (Fig. 6a,b). In contrast, we found no changes in phosphorylation of other calmodulin kinases (CaM, CamKII, CamKK, CaMKIV, Fig. 5a–d). Higher phosphorylation levels of CaMKI in the EC were further corroborated using a specific antibody against pThr177 (Fig. 6c). In addition, using this antibody, we found a similar increase in Thr177 phosphorylation of CaMKI in the phosphoprotein enriched fraction from PRG-1−/− mice and found no signal in the flowthrough-fraction (Fig. 5e). This corroborates the above-mentioned results and points to an effective phosphoprotein purification procedure. Since neuronal hyperexcitation in PRG-1−/− mice required activation of presynaptic LPA2-R (Trimbuch et al. 2009) and additional deletion of this receptor reduced neuronal activity in PRG-1−/− slices (Fig. 3d) and rescued the axon growth deficit in PRG-1−/− explants to WT levels (Fig. 4c,d), we analyzed CaMKI phosphorylation in EC lysates from WT mice. We found lower LPA2-R-deficient background. Here, we found normal CaMKI phosphorylation (Fig. 6d) supporting the idea that normalized neuronal activity in the EC, as present in PRG-1−/−/LPA2-R−/−, rescued axon growth via Ca2+-dependent signal transduction.

Since Ca2+-induced signaling was reported to regulate molecules directly involved in remodeling of the axon’s actin cytoskeleton like LimK1 (Saito et al. 2013), we analyzed their expression and found significantly decreased levels of phosphorylated LimK1/2 while total protein levels of LimK1 were not altered (Fig. 6e,f). LimK1 is critically involved in phosphorylation and thereby deactivation of coflin, which in its active form stimulates actin depolymerization and disruption of actin filaments (Mizuno 2013). Therefore, we further assessed coflin and its phosphorylation levels finding unaltered protein levels but significantly decreased levels of phosphorylated coflin (Fig. 6g,h). This is in line with lower LimK1/2 phosphorylation levels and lower axon growth rates in axons derived from PRG-1−/− slices.

Consequences of Premature Entorhinal Activity for the Adult Brain

To test whether changes in the onset of excitatory activity in assemblies of entorhinal networks also occur in vivo, we recorded spontaneous field potentials in layer II/III of the MEC in living P5 WT and PRG-1−/− mice. As depicted in Figure 7a, by color codes reflecting the multunit electrical activity (MUA) measured by an 8-shank 128-channel electrode, synchronous excitatory network activity was highly present in the EC of PRG-1−/− mice. Quantitative analysis revealed a significantly higher occurrence of discharges and an increased mean firing rate in the MEC of PRG-1−/− mice, confirming the critical role of synaptic bioactive phospholipids in early excitatory network activity in the EC in vivo (Fig. 7a,i–j).

Next, we assessed the impact of premature network activity for the mature perforant path at a structural level in vivo and quantified this projection in adult animals. Using a chemical staining approach of single axons, we labeled the EC perforant projection, which crosses the hippocampal fissure where it can be visualized at the single fiber level (Fig. 7b, black box indicating the hippocampal fissure, and higher magnification of entorhinal axons in the insert). Axon fiber density in PRG-1−/− animals was visibly lower than in WT animals (Fig. 7c). Using unbiased high-resolution stereological methods and serial sections, we assessed the number of axons per animal crossing the hippocampal fissure. To compare the results independently of the animal background, we calculated fiber number per animal as a percentage of control fibers and found a significant lower number of axons in PRG-1−/− mice compared with their WT litters (Fig. 7d). Since additional LPA2-R deletion restored early excitatory activity in the EC and axonal outgrowth to WT levels, we analyzed axon fiber numbers in WT and PRG-1−/− mice on an LPA2-R−/− background (Fig. 7e,f). We found no longer differences as seen in PRG-1−/− mice (Fig. 7g) confirming the effect of premature increase of entorhinal activity on circuit formation up to adult ages.

Discussion

Our study demonstrates that bioactive phospholipids modulate the onset of early neuronal activity in the developing EC and play a critical role in the formation of proper entorhinal–hippocampal connectivity. This was mediated by glutamate-dependent Ca2+-signaling in entorhinal neurons via CaMKII and downstream pathways involving LimK1 and coflin, controlling the stability of the axonal cytoskeleton. Proper appearance of excitatory entorhinal network activity comprising large groups of neurons during critical periods of the development of the entorhinal projection to the hippocampus is under the control of synaptic phospholipid signaling and regulates the amount of fibers of this projection. The general view on early neuronal activity, so far, focused on defects in circuit formation upon its inhibition (Catalano and Shatz 1998; Hanson and Landmesser 2004). Here, we show that premature induction of entorhinal excitatory activity—by only 2 days before its normal appearance—decreased axon growth thereby causing structural alterations in the adult animal.

Phospholipids Modulate Entorhinal Activity Affecting Axon Outgrowth

Spontaneous network activity in the developing brain is a hallmark of young neuronal circuits and is homeostatically regulated (Hanson and Landmesser 2004; Blankenship and Feller 2010). Here, we show that the bioactive phospholipid LPA and its postsynaptic regulatory molecule PRG-1 are key players in this homeostatic control within the entorhinal–hippocampal circuit. While genetic studies suggested that spontaneous neuronal activity was sufficient for embryonic fiber tract development (Molnar et al. 2002), recent studies show that increased glutamatergic levels at the somatodendritic compartment inhibited neurite outgrowth (Yamada et al. 2008; Malyshevskaya et al. 2013). This is in line with our live-imaging analysis of outgrowing fibers showing that enhanced glutamatergic activity slows down axon growth, which could be rescued by blocking neuronal activity. Furthermore, our data do not support a negative effect of
putative higher LPA concentrations in the PRG-1-deficient hippocampus on ingrowing entorhinal axons, which is conceivable from other experimental settings (Campbell and Holt 2003), but argue for the critical importance of the degree of glutamatergic activity.

To prove that the observed reduced axon outgrowth was the direct result of increased excitatory drive of the outgrowing postsynaptic neuron, we performed cell-type-specific PRG-1 deletion in entorhinal neurons that are embedded in an otherwise unaltered environment. Hereby, we could show that altered bioactive synaptic lipid signaling due to lack of postsynaptic PRG-1 and subsequent increased excitatory drive (Trimbuch et al. 2009) in transfected neurons were responsible for the observed reduced axon outgrowth effect.

**Premature Onset of Excitatory Activity Reduced Axon Outgrowth Via Ca2+-dependent Signaling**

It is generally accepted that increased neuronal activity affects gene expression (Greer and Greenberg 2008). To understand the mechanism coupling increased excitatory activity and decreased axon outgrowth on a molecular level, we investigated Ca2+-dependent signal transduction that is activated upon neuronal activity (Wayman et al. 2008). We found a significant phosphorylation increase of CaMKI, but not of other members of the Ca2+/CaM-pathway, which is a critical molecule for axon outgrowth (Ageta-Ishihara et al. 2009). This is in line with recent data showing that phosphorylation of downstream targets of neuronal activity like Ca2+ signaling molecules has to be homoeostatically regulated to assure proper function. Constitutive activation of the CaMKKε leading to hyperphosphorylation of CaMKI (Kaitsuka et al. 2011) or chronic enhancement of CREB activity (Viosca et al. 2009), a downstream target of the CaM kinase, both led to impairment of spatial memory acquisition.

To further understand the downstream Ca2+-signaling up to molecules directly modeling the axon’s actin cytoskeleton, we analyzed EC-specific phosphorylation levels of LimK1 which is a target of the Ca2+/calmodulin-dependent protein kinases (Takemura et al. 2009; Saito et al. 2013) and inactivates coflin activity via phosphorylation (Mizuno 2013). Coflin negatively regulates actin dynamics and destabilizes axons via actin depolymerization (Mizuno 2013; Saito et al. 2013). In fact, we could show that both LimK1/2 and coflin phosphorylation levels were decreased which results in an increased coflin activity and a negative effect on axon outgrowth (Mizuno 2013; Saito et al. 2013). However, as reported in other systems (Pandey et al. 2007), it is well feasible that in parallel to a reduction of LimK1/2-mediated coflin phosphorylation, a direct, Ca2+-dependent coflin dephosphorylation occurs, which results in alterations of axon outgrowth alike.

**Premature Excitatory Activity Affects Entorhinal–Hippocampal Networks in Adult Animals**

Since dentate gyrus innervation follows a precise spatiotemporal developmental pattern during the early postnatal period (Frotscher et al. 2000), our data imply alterations of the proper connectivity between EC and hippocampus. Since underlying mechanisms inducing premature neuronal activity in the EC occur in the postnatal period, other dentate gyrus input systems developing at earlier stages such as the septo-hippocampal projection (Linke and Frotscher 1993) playing an important role for hippocampal function (Hangya et al. 2009; Vandecasteele et al. 2014) are supposed to remain unaffected. In sum, our data suggest that synaptic LPA signaling via presynaptic LPA2Rs and under physiological control of the postsynaptic PRG-1 molecule regulates early entorhinal excitatory activity affecting neuronal circuit formation important for memory function in adult animals. Since the early postnatal processes studied here in the mouse brain are equivalent to the development in primates (Amaral et al. 2014) and hippocampus-dependent memory emerges during early childhood concomitant with the maturation of hippocampal circuits (Lavenex and Banta Lavenex 2013), our findings imply an important role of proper onset of entorhinal excitatory activity during human brain development involving bioactive lipid signaling, and call for caution in affecting this process by any external intervention.

**Supplementary Material**

Supplementary data are available at Cerebral Cortex online.

**Authors’ Contributions**

J.V., R.N. designed the experiments and wrote the paper. J.V., S.K., B.L., H.J.L., and R.N. contributed to the experimental design, supervised the experiments and the data analysis or editing. J.A. and J.C. provided antibodies and transgenic animals, respectively. L.S., J.-W.Y., P.U., J.C., U.S., A.P., A.Pr., and B.S.B. were involved in the different experiments.

**Notes**

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