ORIGINAL ARTICLE

Development of inhibitory synaptic inputs on layer 2/3 pyramidal neurons in the rat medial prefrontal cortex

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Received: 31 May 2017 / Accepted: 28 December 2017
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Abstract

AQ1 Inhibitory control of pyramidal neurons plays a major role in governing the excitability in the brain. While spatial mapping **AQ2** of inhibitory inputs onto pyramidal neurons would provide important structural data on neuronal signaling, studying their distribution at the single cell level is difficult due to the lack of easily identifiable anatomical proxies. Here, we describe an approach where in utero electroporation of a plasmid encoding for fluorescently tagged gephyrin into the precursors of pyramidal cells along with iontophoretic injection of Lucifer Yellow can reliably and specifically detect GABAergic synapses on the dendritic arbour of single pyramidal neurons. Using this technique and focusing on the basal dendritic arbour of layer 2/3 pyramidal cells of the medial prefrontal cortex, we demonstrate an intense development of GABAergic inputs onto these cells between postnatal days 10 and 20. While the spatial distribution of gephyrin clusters was not affected by the distance from the cell body at postnatal day 10, we found that distal dendritic segments appeared to have a higher gephyrin density at later developmental stages. We also show a transient increase around postnatal day 20 in the percentage of spines that are carrying a gephyrin cluster, indicative of innervation by a GABAergic terminal. Since the precise spatial arrangement of synaptic inputs is an important determinant of neuronal responses, we believe that the method described in this work may allow a better understanding of how inhibition settles together with excitation, and serve as basics for further modelling studies focusing on the geometry of dendritic inhibition during development.

Keywords Development · Cerebral cortex · GABA · Gephyrin · Synaptogenesis

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00429-017-1602-0>) contains supplementary material, which is available to authorized users.

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Introduction

Integration of excitatory synaptic inputs in principal neurons is tightly controlled by inhibitory microcircuits throughout the entire central nervous system (CNS). This modulation is primarily achieved via synaptic contacts formed by GABAergic and glycinergic inhibitory interneurons onto the dendritic tree and soma of principal cells. The distribution of inhibitory contacts through the neuronal arbor together with their spatial relation to excitatory synapses are critical determinants of the somatic membrane potential and neuronal spiking patterns (Hao et al. 2009; Kaila et al. 2014; Kubota et al. 2016; Müller et al. 2012). Therefore, mapping of excitatory and inhibitory terminals at the single cell level provides important morphological information on spatial factors that are involved in neuronal computation (Spruston 2008).

In the rodent cerebral cortex, only a very small number of inhibitory synapses are present at birth, with the peak

phase of synaptogenesis occurring between the second and fourth postnatal weeks (De Felipe et al. 1997; Micheva and Beaulieu 1996). During this period, there is a well over tenfold, cortical layer-specific increase in the number of both excitatory and inhibitory synapses, and this intense growth is associated with marked changes in the proportion of excitatory vs. inhibitory contacts over time (De Felipe et al. 1997). Despite the importance of inhibitory control of pyramidal cells, little is known about the spatiotemporal development of inhibitory synaptic inputs at the single cell level. This is mainly related to the fact that, in contrast to excitatory contacts where dendritic spines can be used as anatomical proxies, no structures exist for easily identifying inhibitory inputs on dye- or fluorescently-labelled individual neurons. Therefore, GABAergic synaptogenesis in early postnatal rodent brain has mainly been studied using volumetric electron microscopy, and the data is often given per volume of tissue without regard to the identity of the postsynaptic cell (De Felipe et al. 1997; Micheva and Beaulieu 1996). While three-dimensional electron microscopic reconstruction of the neuronal dendritic arbor along with its cell specific synaptic inputs is technically feasible, this approach is extremely labor intensive. Hence, although some quantitative information on the spatial aspects of inhibitory synaptic inputs onto neurons exists in the adult brain (Gulyás et al. 1999; Klausberger and Somogyi 2008; Megías et al. 2001), very little data is available during development.

Recent advances in molecular tools now allow us to detect synaptic proteins, enabling specific labelling of synaptic contacts with high accuracy and relative ease. The inhibitory scaffolding protein gephyrin has proven to be particularly useful in this respect, enabling specific labelling of synaptic contacts with high accuracy and relative ease. Gephyrin molecules form dense clusters only at inhibitory postsynaptic locations, where they compose a signaling scaffold that gathers both postsynaptic receptors and transduction molecules at synaptic sites (Tyagarajan and Fritschy 2014). Recent studies have convincingly demonstrated that in utero electroporation of fluorescently tagged gephyrin into the progenitors of specific neuronal populations is a highly reliable tool for localizing inhibitory synapses on the dendritic arbor of single cells even in adult animals (Chen et al. 2012; van Versendaal et al. 2012).

The primary goal of the present study was to establish a method that allows identification of inhibitory synaptic inputs on pyramidal neurons at the single cell level with relative ease. A related secondary aim was to apply this method to describing the spatio-temporal pattern of inhibitory synaptogenesis on developing pyramidal neurons during the first postnatal month. In these experiments, we focused on the rat medial prefrontal cortex (mPFC), a cortical region that is considered as the functional equivalent of the human prefrontal cortex (Uylings et al. 2003), which is

implicated in higher order cognitive and emotional processing (Tsujimoto 2008). We first used immunohistochemistry to visualize endogenous gephyrin, to estimate the density of GABAergic synapses at different developmental stages in the rat mPFC. We then created a method to identify inhibitory inputs on single pyramidal neurons using in utero electroporation (IUE) of a plasmid encoding for fluorescently tagged gephyrin into layer 2/3 pyramidal neurons. Combining this approach with single cell post hoc injection of the fluorescent dye Lucifer Yellow, which reveals the detailed architecture of the dendritic arbor, allowed the simultaneous detection of inhibitory synapses along with the dendritic spines as anatomical proxies for excitatory synapses on the same dendritic segments. Using this technique, we were able to quantify the developmental emergence of glutamatergic and GABAergic synapses on the basal dendritic tree of single layer 2/3 pyramidal neurons in the rat mPFC during the first postnatal month. Together, these observations offer a useful tool to trace excitatory and inhibitory synaptogenesis at the single cell level in different brain areas which, in turn, may provide a better understanding regarding the functional integration of synaptic inputs in pyramidal neurons.

Materials and methods

Animals

All experiments were conducted according to the guidelines of the Swiss Federal Veterinary Office and they were approved by the cantonal Animal Welfare Committee of Geneva. Wistar rats (Charles River, Arbresle, France) were housed and bred in the animal facilities under a 12 h light/dark cycle and temperature-controlled (22 ± 2 °C) conditions. Food and water were available ad libitum.

In utero electroporation and expression Vectors

In utero electroporation was performed according to Saito and Nakatsuji (Saito and Nakatsuji 2001) with minor modifications. Briefly, timed pregnant Wistar rats with E17.5 embryos were anaesthetized with isoflurane (3.5% induction, 2.5% during the surgery), and uterine horns were successively exposed after a midline laparotomy. All embryos were injected with 2 µl of tdTomato-Gephyrin (TomGeph) plasmid DNA solution [2 µg/µl, prepared in 0.9% NaCl, 0.3 mg/ml Fast Green (Sigma-Aldrich, St. Louis, MO)] into the lateral ventricle through the uterine membrane. The tdTomato-Gephyrin construct was engineered by substituting eGFP with the fluorescent marker tdTomato using the *NheI* and *XhoI* restriction sites present in the peGFP-gephyrin vector (Lardi-Studler et al. 2007; Tyagarajan et al. 2011a, b). Cytomegalovirus-derived expression from this

new construct produces an N-terminal red fluorescently tagged gephyrin fusion protein. Cells were electroporated by holding the head of each embryo between tweezer-style circular electrodes (7 mm diameter, Harvard Apparatus) across the uterus wall while 5 pulses (50 V, 50 ms duration) were delivered with a square-wave generator (ECM 830; Harvard Apparatus). To optimize the number of electroporated cells, the interval between the pulses was adjusted to be either 950 ms (experiments where clusters were colocalized with the Lucifer Yellow filled dendrites) or 100 ms (all other experiments). The uterine horns were returned into the abdominal cavity, the wall and skin were sutured, and the embryos were allowed to resume normal development. For pain medication, the mother was given Temgesic (0.03 mg/kg s.c. injection, Reckitt Benckiser, Wallisellen) and Dafalgan (0.5 g/l in drinking water, Bristol-Myers Squibb SA, Steinhausen). Baytril (1.5% in 100 ul i.m., Bayer Vital GmbH, Leverkusen) was used as an antibiotic.

Iontophoretic post hoc single cell injections

Animals were sacrificed at defined time points by an intraperitoneal overdose of pentobarbital (200 mg/kg, Inresa Arzneimittel GmbH, Freiburg) followed by a cardiac perfusion with a 4% paraformaldehyde and 0.125% glutaraldehyde solution (pH 7.4). Brains were removed and postfixed for 2 h in 4% paraformaldehyde. Coronal sections of 300 µm thickness were cut on a vibratome in ice-cold phosphate-based saline solution (PBS, pH 7.4). Individual slices were then mounted into an injection chamber filled with ice-cold PBS and placed on the fixed stage of an Olympus microscope equipped with a micromanipulator. Electroporated layer 2/3 pyramidal neurons (approximately 200–300 per 300 µm thick cortical coronal section) were identified under fluorescence illumination (X-Cite 120, Lumen Dynamics Group Inc. Mississauga, ON) and a 40× water immersion objective. For the control experiments the non-electroporated cells were visualized by counterstaining the slices 5 min at +4 °C with 0.1% methylene blue (Bichsel, Interlaken). Single cells were targeted using sharp microelectrodes and loaded iontophoretically with a 0.4% Lucifer Yellow solution (Sigma-Aldrich) applying a negative current of 70 nA, until the dendrites were fluorescing brightly. For each animal 2–3 slices were labelled each with 6–12 randomly chosen cells.

Immunohistochemistry

The iontophoretically labelled slices were preincubated for 1 h in a PBS solution containing sucrose (5%), bovine serum albumin (2%), Triton X-100 (1%) and sodium azide (0.1%), followed by 48 h at room temperature with the anti-Lucifer Yellow antibody (rabbit IgG, Cat.No. A5750, Invitrogen,

Carlsbad, CA; 1:4000 dilution). Slices were then rinsed in PBS solution and incubated for an additional 24 h with Alexa conjugated secondary antibodies (Invitrogen; 1:1000).

For the anti-gephyrin immunostaining brains were dissected in ice-cold PBS, and immediately frozen in isopentane (Sigma-Aldrich, puriss. p.a. ≥ 99.5%) placed on dry ice (− 70 °C). After at least one night at − 20 °C, cryosections of 20 µm thickness were cut onto an electrostatic adhesion slide, which was then lifted in room temperature for 30 s and returned in − 20 °C until the immunostaining. Tissue was fixed by immersing the slides in − 20 °C methanol for 30 s and rinsed with PBS. Sections were preincubated for 1 h in room temperature in a PBS solution containing bovine serum albumin (0.5%), Triton X-100 (0.3%), and sodium azide (0.1%) and for further 24 h at +4 °C with the primary antibody (anti-gephyrin, mouse monoclonal hybridoma supernatant mAb7a, Cat. no. 147021, Synaptic Systems, Göttingen; 1:500). Sections were then rinsed with PBS and incubated with Alexa conjugated secondary antibodies (Invitrogen; 1:1000) at room temperature for 2 h in a PBS solution containing bovine serum albumin (0.5%) and sodium azide (0.1%).

For the anti-VGAT staining animals were perfused transcardially with 4% paraformaldehyde, the brains were post-fixed overnight and then embedded stepwise in 30% sucrose. Cryosections of 7 µm thickness were mounted on electrostatic adhesion slides, and kept in − 20 °C until the immunostaining. Sections were preincubated for 2 h in room temperature in a PBS solution containing sucrose (5%), bovine serum albumin (2%), Triton X-100 (1%) and sodium azide (0.1%) and for further 72 h at room temperature with the primary antibody (anti-VGAT, rabbit polyclonal antiserum Cat. no. 131002, Synaptic Systems; 1:2000). Sections were then rinsed with PBS and incubated with Alexa conjugated secondary antibodies (Invitrogen; 1:1000) at room temperature for 24 h. Slices were mounted and coverslipped using Immumount (Thermo Scientific, Pittsburgh, PA) and stored at +4 °C until analysis.

Confocal laser scanning microscopy, image analysis and dendritic reconstruction

A confocal microscope (LSM 700 Zen, Zeiss) equipped with a 63× oil-immersion objective (1.4 NA) was used for image acquisition. The pinhole was set at 1 Airy unit and wavelengths were separated to sequentially imaged channels using the full intensity range of detection. For the Lucifer Yellow filled cells stacks of 15–35 confocal sections (1084 × 1084 pixels, 90 nm per pixel, 380 nm intervals) were acquired. In each age group, the imaging settings for TomGeph were kept constant across all treatments. For the anti-gephyrin immunostaining we used a single plane in the middle of the slice (360 × 360 pixels, 90 nm per pixel),

and for the VGAT-colocalization a stack of 15–20 images (540 × 540 pixels, 90 nm per pixel, 380 nm intervals).

The gephyrin immunostaining was analysed using a custom written algorithm in MetaMorph (MolecularDevices, Sunnyvale, CA). The detection parameters (minimum and maximum width, intensity above the local background) were first optimized with a test set of images covering the whole range of staining intensities (see Electronic Supplementary Table 1 for detailed description of the algorithm). Once set, detection parameters were kept identical for all age groups analysed.

Colocalization of electroporated TomGeph clusters with VGAT clusters was quantified manually with the Neurolucida confocal image stack module in 3D image stacks taken from the cortical layer 1. First, offsets for both channels were optimized to give the best contrast between the clusters and the background. Then, with the channel for VGAT turned off, randomly chosen TomGeph clusters were labelled for analysis. Clusters were defined to colocalize with a VGAT cluster if they either overlapped, or their edges were in direct contact with each other. As a control, the same analysis was repeated with identical settings, but with one of the channels rotated by 90°, giving the proportion of random colocalization. Clusters located on the surface of the slice and those landing outside the tissue after channel rotation were excluded from the analysis.

For the proof of principle experiments, TomGeph electroporated cells were compared in the same brain slices with their neighbouring non-electroporated control cells. After Lucifer Yellow injection, second order dendrites were imaged for spine analysis using LSM700 confocal microscope and 63× oil-immersion objective, and the spines were counted manually in 3D image stacks. The reconstruction of the whole dendritic arbour was done using a computer-based Neurolucida system with a 100× oil-immersion objective on a Nikon microscope (Nikon Corporation, Tokyo, Japan).

For the quantification of gephyrin clusters on single neurons, Lucifer Yellow filled cells were traced manually in 3D using the Neurolucida confocal image stack module software (v10.53.1., Microbrightfield, Williston, VT). Cells were traced in the image stacks starting from the soma and following the dendrites as far as they could be reliably analyzed. Each gephyrin cluster and dendritic spine was marked on the tree during the tracing. Gephyrin clusters on dendritic shafts and spines were quantified on raw images and defined as clusters of at least four adjacent pixels (2 × 2, 0.18 μm diameter) detected on at least two consecutive *z* planes and displaying a minimal intensity of 3% of the intensity range (4096 Gy levels). The mean intensity of the smallest cluster detected using these parameters was 5% of the intensity range, and the mean intensity of a halo region drawn around this cluster was 0.3% of the intensity range. In our images, the mean intensity of the median-size cluster represented

26% of the intensity range, while the mean intensity of the halo region drawn around it was 0.8%. We found no saturated pixels in the cluster we have detected (max pixel intensity: 98.8% of the intensity range). When a detected cluster was co-localized at its brightest *z* plane with Lucifer Yellow fluorescence, it was considered to belong to the dendrite or spine of the injected neuron. Otherwise, it was excluded from the analysis. Only brains with a low number of electroporated cells were included in the analysis, to avoid cases of fluorescent noise coming from the neighbouring cells. Analyzed dendrites were required to have a visible intracellular TomGeph background, to ensure that even the dimmest puncta would be detectable. From the tracings, data were extracted using Neurolucida Explorer (v10.53.1.) and MATLAB (R2012a). In Scholl analysis, for each cell, bins with less than 5 μm dendritic length were excluded. Similarly, for all analysis comparing single dendritic segments, a minimum length of 5 μm was required.

Statistical analysis

In experiments using IUE, the experimental unit was the cell. For each age group, 18–22 cells were analyzed. To exclude potential biases related to interindividual and/or between litter differences, these cells were obtained from 5 to 8 animals which originated from 4 separate litters in each group. The total dendritic length traced per group was between 3467.4 and 5870.5 μm. In proof of principle experiments, to ascertain that the electroporation of fluorescently tagged gephyrin does not affect neuronal development per se, neighbouring electroporated and non-electroporated cells were sampled from 3 animals which originated from 3 separate litters, and the experimental unit was the cell (*n* = 17–21 cells for dendritic tracing, and 11 cells for spine densities). For the anti-gephyrin immunostaining, the experimental unit was the animal. For each animal (*n* = 3), four brain sections were sampled for all cortical layers. No difference was found between the left and the right hemisphere, compared layer by layer (*p* > 0.685 for each age group; two-way ANOVAs with Sidak's post hoc tests, factor 1: hemisphere: *p* > 0.12 × factor 2: developmental stage (P5, P10, P15, P20, P30): *p* < 0.0001; interaction: *p* > 0.751). Statistics were done with GraphPad Prism (version 6.03, La Jolla, CA) All values are presented as the mean ± the standard error of mean (SEM), unless otherwise indicated. Normality was tested for each distribution (D'Agostino-Pearson normality test). For two distributions comparison, two-tailed Student's *t* test was performed when data was Gaussian, otherwise Mann–Whitney test was used. For multiple comparisons of Gaussian distributions, statistical significance was determined using one- or two-way ANOVA followed by Tukey's post hoc tests (corrected for multiple comparisons). Kruskal–Wallis test followed by Dunn's post hoc tests (corrected for multiple comparisons)

was used when the data distribution was significantly different from a Gaussian distribution. For all tests, α was set to 5% and $p < 0.05$ was considered as statistically significant.

Results

Cortical layer-specific developmental increase in gephyrin expression in the rat medial prefrontal cortex

Based on the previously described time frame of inhibitory synaptogenesis in the cerebral cortex (De Felipe et al. 1997; Micheva and Beaulieu 1996), we first aimed to describe quantitative aspects of GABAergic

synaptogenesis in the different layers of the early postnatal mPFC by staining histological sections of the rat mPFC with a well-characterized antibody that recognizes the GABAergic postsynaptic scaffolding molecule gephyrin (Fig. 1). As seen on Fig. 1a, gephyrin immunohistochemistry results in a bright punctiform staining pattern with age- and cortical layer-specific differences in the density of the punctiform gephyrin clusters. Quantitative analysis of the gephyrin-positive clusters confirms a massive increase in the number of GABAergic synapses during the first postnatal month in all cortical layers (Fig. 1b–d). At P5, we found that gephyrin cluster density was several fold higher in cortical layer (L) 1 when compared with other cortical layers. The largest increase in the number of gephyrin-positive clusters over

Fig. 1 Development of gephyrin immunoreactivity in the rat medial prefrontal cortex during the first postnatal month. **a** Representative single plane confocal images showing the cortical layer-specific development of immunostaining against endogenous gephyrin in the rat mPFC between postnatal days 5 and 30. **b** Quantification of the punctate staining expressed as number of gephyrin clusters per 100 μm^2 . * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$, $n = 3$ animals per

age group. **c** Schematic drawing showing the part of mPFC that was sampled for analysis [modified from (Paxinos and Watson 1998)]. **d** Example of the analysis. Clusters were detected using a custom made algorithm (Electronic Supplementary Table 1) which first binarized the image based on local differences in intensity and then counted the number of openings corresponding to single gephyrin clusters. Scale bars: 5 μm

the first postnatal month occurred in cortical L2/3, with an approximately 17-fold increase between postnatal days (P) 5 and P30 (growing from 2.6 ± 0.5 to 48.1 ± 2.7 clusters per $100 \mu\text{m}^2$), while the smallest amplitude of change was detected in L1 (95% increase between P5 and P30, from 28.7 ± 4.0 to 57.2 ± 4.3 clusters per $100 \mu\text{m}^2$). Cluster density increased significantly between P5 and P10 in L1 ($p = 0.026$), and between P10 and P15 in all cortical layers ($p = 0.0007$ in L1, $p < 0.0001$ in L2/3, $p = 0.0005$ in L5/6, two-way ANOVA with Tukey's post hoc tests, factor 1: cortical layer: $p < 0.0001 \times$ factor 2: developmental stage: $p < 0.0001$; interaction: $p = 0.114$). After P15, no further

significant change was observed. In age groups from P5 to P20, gephyrin cluster density was significantly higher in L1 compared to the other layers ($p < 0.001$ for each age group) while at P30, only L5/6 cluster density remained significantly smaller than in L1 ($p = 0.0035$), and no differences were found for any age group between L2/3 and L5/6 (two-way ANOVA with Tukey's post hoc tests, factor 1: cortical layer: $p < 0.0001 \times$ factor 2: developmental stage: $p < 0.0001$; interaction: $p = 0.114$). These data indicate that the density of GABAergic synapses undergoes a massive increase in all cortical layers during the first postnatal weeks.

Fig. 2 Combining in utero electroporation of TomGeph with post hoc iontophoretic injection of Lucifer Yellow allows the analysis of GABAergic synapses on the dendritic tree of single pyramidal neurons, without perturbing their normal development. **a** Iontophoretic post hoc injection of Lucifer Yellow in single electroporated neurons reveals the whole dendritic arbor with high resolution. **b** Single plane image with orthogonal views showing an example of a fluorescent gephyrin cluster colocalizing on the dendritic shaft. **c** Typical example of a gephyrin cluster located on a spine head. **d** Experimental timeline. In utero electroporation (IUE) was done at embryonic day 17.5 and the pups were born as normal on postnatal day (P) 0. Animals were fixed at defined developmental time points (P5–30), and single cells were filled iontophoretically with Lucifer Yellow. Slices were then imaged using confocal microscope, and image stacks were traced manually. **e** Electroporated TomGeph molecules form clusters that colocalize with the GABAergic presynaptic vesicular GABA transporter VGAT. Altogether 93.4% of the 935 randomly chosen gephyrin clusters colocalized with a presynaptic terminal. **f** Example of the tracing. A three-dimensional reconstruction of the basal dendritic tree was reconstructed by tracing the dendrites starting from the soma. Each dendritic spine was marked, and the gephyrin clusters were separately labelled on spines (green cross) and on the dendritic shaft (green circles). The size of the cluster was visually estimated by the diameter of the respective marker. **g** A maximum intensity projection showing the risk of false results in the colocalization analysis after image projection. In the original image stacks, some clusters were judged to colocalize with the Lucifer Yellow injection (white arrows), whereas others were not (blue arrows). After projecting the image stack, some clusters will falsely appear to colocalize with the cell. See Supplementary Fig. 1 for the original image stack. **h** No significant differences were found in the total dendritic length in either apical ($p=0.52$) or basal ($p=0.26$) dendrites between the TomGeph electroporated cells and the neighbouring non-electroporated control cells ($n=17$ –21 cells from three animals). **i** The number of dendritic branching points were not statistically different in apical ($p=0.35$) or basal ($p=0.74$) dendrites. **j** No significant differences were found in spine dendrites between the TomGeph electroporated cells and the neighbouring non-electroporated control cells ($p=0.40$, $n=11$ cells from three animals). Scale bars: **a** 20 μm ; **b, c, f, g** 1 μm ; **e** 500 nm

In utero electroporation of a fluorescently tagged gephyrin reveals GABAergic synapses on pyramidal neurons without perturbing neuronal development

Although the immunostaining against endogenous gephyrin gives a general view to the synaptogenesis occurring in the different layers of the mPFC, it does not differentiate between the possible postsynaptic targets. To specifically study inhibitory inputs on principal neurons, we performed IUE of a plasmid encoding for fluorescently tagged gephyrin at E17.5 into precursors of layer 2/3 mPFC neurons, and the detailed dendritic arbour of these electroporated principal cells were revealed by iontophoretic injection of Lucifer Yellow at defined stages of postnatal development (Fig. 2a–d). We have previously shown that the electroporation procedure per se does not interfere with the normal pattern of dendritic arbor and spinogenesis (Fiumelli et al. 2013). Here, we first conducted a series of proof of principle experiments to confirm that the expression of exogenous fluorescently tagged gephyrin indeed reveals GABAergic synapses, and that this

genetic manipulation does not impair the normal patterns of dendritic arbor development in pyramidal neurons.

To confirm that the overexpressed fluorescent gephyrin incorporates into the GABAergic synapses, we stained brain sections from electroporated P20 animals against the presynaptic GABAergic marker VGAT. In confocal image stacks, a total of 935 fluorescent TomGeph clusters were randomly chosen for analysis. Out of these, altogether 96.4% colocalized with a VGAT cluster (Fig. 2e). This was significantly different from the level of random colocalization ($p<0.0001$, Fisher's exact test), which was determined to be 75.8% by repeating the analysis with the same settings after rotating one of the channels by 90°.

Quantitative analysis of neuronal dendritic arbour dimensions in Lucifer Yellow-injected layer 2/3 principal cells using the NeuroLucida software (MBF Bioscience, Williston, Vermont, USA) revealed no statistically significant difference between electroporated and non-electroporated cells in either apical (total dendritic length: 1385 ± 119 vs. 1268 ± 137 μm ; $p=0.52$, $n=17$ –21 cells, unpaired t test) or basal dendrites (1221 ± 86 vs. 1083 ± 83 μm ; $p=0.26$, unpaired t test, Fig. 2h). Furthermore, no significant differences were found in the number of dendritic branching points in apical (15.1 ± 1.2 vs. 13.5 ± 1.2 , electroporated vs. non-electroporated cells, respectively; $p=0.35$, unpaired t test) or in basal dendrites (12.6 ± 1.1 vs. 11.4 ± 0.9 ; $p=0.74$, Mann–Whitney test, Fig. 2i). We also quantified spine density in basal dendrites of TomGeph electroporated and the neighbouring non-electroporated cells (120 ± 3 and 120 ± 2 per 100 μm , respectively) and found no statistically significant differences ($p=0.40$, unpaired t test, $n=11$ cells per group, Fig. 2j). These results thus demonstrate that IUE of fluorescently tagged gephyrin reliably labels GABAergic post-synaptic sites and does not alter the morphological properties of L2/3 pyramidal neurons.

Time course of GABAergic synaptogenesis onto basal dendrites of pyramidal neurons in the early postnatal period

To describe the spatiotemporal development of GABAergic synaptic inputs at the single cell level, we focused on basal dendritic tree of layer 2/3 pyramidal neurons in the rat mPFC and analyzed the distribution of fluorescent gephyrin clusters between P10 and P30 (see “Materials and methods”). Spine density increased nearly linearly across all sampled age groups, from 23 ± 1 per 100 μm at P10 to 160 ± 8 per 100 μm at P30 ($p<0.001$ for each age group, one-way ANOVA with Tukey's post hoc tests, Fig. 3a). The mean gephyrin cluster density on dendritic shafts increased significantly from 4.1 ± 0.5 per 100 μm at P10 to 12.8 ± 0.5 per 100 μm at P15 (207% increase, $p=0.0068$, Kruskal–Wallis test with Dunn's post-hoc tests), and further to 21.2 ± 1.4 per

Fig. 3 Development of gephyrin clustering on basal dendrites of layer 2/3 pyramidal neurons of rat mPFC during the first postnatal month. **a** Dendritic spine density, expressed as spine number per 100 μm , from P10 to P30. $^{***}p < 0.01$. Cluster density, expressed as cluster number per 100 μm , for gephyrin clusters located on dendritic shafts (**b**) and spines (**c**), in the same dendritic segments as in **a**. $^{**}p < 0.01$; $^{****}p < 0.0001$. Sholl analysis showing density of dendritic spine (**d**), gephyrin cluster on dendritic shaft (**e**) and gephyrin cluster on dendritic spine (**f**) as a function of distance from the soma. Spine den-

sity was significantly affected by the distance from the soma in all age groups ($p < 0.0001$) except P10 ($p = 0.84$). The density of gephyrin clusters on the dendritic shaft was also significantly affected by the distance from the soma at P15 ($p < 0.0001$) and at P30 ($p < 0.01$), but not at P10 ($p = 0.36$) or at P20 ($p = 0.06$). The density of gephyrin cluster located on spines was significantly affected by distance at P15, P20, and P30 ($p < 0.0001$), but not at P10 ($p = 0.11$). For each age group, n number is indicated in the insert

100 μm at P20 (66% increase from P15 to P20, $p = 0.0023$), but in contrast to dendritic spines, no further significant change was observed at P30, when the mean density was $20.9 \pm 1.1 \mu\text{m}^{-1}$ (P20 vs. P30, $p > 0.99$) (Fig. 3b). Similar to the shaft clusters, the mean density of gephyrin clusters on spines increased significantly from 1.1 ± 0.3 per 100 μm at P10 to 5.8 ± 0.5 per 100 μm at P15 (414% increase, $p < 0.0001$, one-way ANOVA with Tukey's post-hoc tests),

and to 10.6 ± 0.8 per 100 μm at P20 (83% increase from P15 to P20, $p < 0.0001$), with no statistically significant change to P30 (9.5 ± 1.1 per 100 μm at P30; P20 vs. P30: $p = 0.76$) (Fig. 3c).

To have a more detailed spatial view of the dendritic spine and gephyrin cluster densities on neuronal dendrites, we performed Sholl analyses where the cluster density is counted in concentric segments around the cell body

(Sholl 1953) We found that dendritic spine density was significantly affected by the distance from the soma in P15–P30 animals, with the distal dendrites having a higher spine density than the most proximal parts ($p < 0.0001$ for each age group). However, such effect was not observed in younger animals (P10; $p = 0.83$, Kruskal–Wallis tests, Fig. 3d), where the spine density was not affected by the distance from the soma ($p = 0.70$). The density of gephyrin clusters on the shaft was less strikingly affected by the distance from the soma. This was significant at P15 ($p < 0.0001$) and P30 ($p = 0.0065$), but not at P10 ($p = 0.36$) or at P20 ($p = 0.07$) (Kruskal–Wallis tests, Fig. 3e). On the other hand, the density of gephyrin clusters on spines seemed to largely follow the spatial densities of the spines themselves, with a significant effect of distance at all developmental stages between P15 and P30 stages ($p < 0.0001$ for each age group), but not at P10 ($p = 0.11$) (Kruskal–Wallis tests, Fig. 3f). These observations indicate that a spatial organization of inhibitory and excitatory synaptic contacts emerges between P10 and P15 in the layer 2/3 pyramidal neurons of the rat mPFC.

We also investigated whether the density of dendritic spines on a given cell might be correlated with the respective density of gephyrin clusters on the shaft. At the whole cell level, no statistically significant correlations were found between the two parameters ($p > 0.07$ for each age group, Pearson or Spearman correlation). Next, we observed that the mean size of gephyrin clusters remained relatively stable during development (Fig. 4). The mean diameter of gephyrin clusters on dendritic shaft was $0.46 \pm 0.02 \mu\text{m}$ at P10, $0.46 \pm 0.01 \mu\text{m}$ at P15, $0.45 \pm 0.01 \mu\text{m}$ at P20 and $0.44 \pm 0.01 \mu\text{m}$ at P30 (Fig. 4a). The shapes of the size distributions were not significantly different ($p > 0.13$, Kolmogorov–Smirnov tests). The mean diameter of gephyrin clusters on the spines was $0.41 \pm 0.01 \mu\text{m}$ at P10, $0.39 \pm 0.01 \mu\text{m}$ at P15, $0.36 \pm 0.01 \mu\text{m}$ at P20 and $0.36 \pm 0.01 \mu\text{m}$ at P30 (Fig. 4b). The distributions at P20 and P30 were significantly different compared to the values at P10 ($p = 0.037$ and $p = 0.0025$ for P20 and P30, respectively). For both types of gephyrin clusters, in each age group 80–94% of clusters had a diameter between 0.3 and 0.5 μm .

Finally, we focused our attention on putative dually innervated spines, based on gephyrin detection in dendritic spines. We found that the proportion of gephyrin clusters located on dendritic spines increased during development, with the median values increasing significantly from 11.3% at P10 to 32.9% at P20 ($p = 0.017$, Kruskal–Wallis test with Dunn's multiple comparisons) (Fig. 4c). On the other hand, we also analysed the proportion of spines carrying a gephyrin cluster, which indicates a potential dual innervation by a GABAergic terminal (Fig. 4d). The median percentage of spines with a gephyrin cluster increased significantly from P10 to P20 (2.9 and 9.4%, respectively, $p = 0.0074$), and then

decreased again to P30 (6.3%, $p = 0.034$) (Kruskal–Wallis test with Dunn's multiple comparisons).

Discussion

The spatial distribution of excitatory and inhibitory synaptic inputs at the level of single neurons is of fundamental importance to the information processing that mediates nervous system function. Here, we combined in utero electroporation of fluorescently tagged gephyrin into precursors of layer 2/3 pyramidal neurons with the iontophoretic injection of Lucifer Yellow into these cells in the postnatal period, and showed that this approach can reliably and specifically detect GABAergic synapses on the dendritic arbour in the in vivo environment. By providing detailed analysis of the spatial distribution of gephyrin clusters over the basal dendritic arbour, these results constitute, to our knowledge, the first spatiotemporal description of GABAergic synaptic input development on pyramidal neurons during brain maturation.

Our experimental method allowed us to identify simultaneously excitatory and inhibitory postsynaptic sites of contacts at the single cell level, using dendritic spines and gephyrin clusters, respectively, as their anatomical proxies. Previous morphological work on developmental synaptogenesis in the central nervous system has mostly assessed the density of synaptic contacts in a given volume of tissue, with knowledge neither on the identity of the postsynaptic cells nor on the subcellular localization of the synaptic contacts (De Felipe et al. 1997; Micheva and Beaulieu 1996). While this information can be obtained from electron microscopic investigations, reconstructing a whole pyramidal neuron or even a short dendritic segment is extremely time-consuming. More recent studies have taken advantage of combining fluorescently tagged gephyrin with an intracellular soluble fluorescent protein to image GABAergic synapses either in vivo (Chen et al. 2012; van Versendaal et al. 2012) or in vitro using organotypic cultures (Flores et al. 2015). In our investigations, we found that a much better signal-to-noise ratio is achieved in fixed tissue with the iontophoretic intracellular injection of electroporated cells using a fluorescent dye, such as Lucifer Yellow. This method allows having sparse labelling of single cells, needed for precise tracing of dendritic branches. When the injected cells are imaged into confocal stacks, a 3D model of gephyrin clusters on the dendritic tree can be reconstructed. Furthermore, the good resolution of Lucifer Yellow injected cells is ideal for analysing dendritic spines, and the spatial arrangement of gephyrin clusters with respect to spines can reliably be studied in detail. One potential caveat to consider when using dendritic spines as anatomical proxies to detect excitatory synapses is that some excitatory synapses may also contact directly dendritic shafts (Megias et al. 2011). Although

Fig. 4 Size distribution of gephyrin clusters during development and proportion of gephyrin clusters located on dendritic spines. Cumulative size distributions for gephyrin clusters on dendritic shafts (**a**) and on dendritic spines (**b**). The shapes of the distributions at P15, P20 and P30 were pairwise compared to the distribution at P10 and no significant differences were found ($p > 0.13$ for all groups). For gephyrin clusters located on dendritic spines, the mean diameter was $0.41 \pm 0.01 \mu\text{m}$ at P10, $0.39 \pm 0.01 \mu\text{m}$ at P15, $0.36 \pm 0.01 \mu\text{m}$ at P20 and $0.36 \pm 0.01 \mu\text{m}$ at P30. The distributions at P10 and P15 were not significantly different ($p = 0.68$, Kolmogorov–Smirnov rank test), but

both at P20 and P30 they differed significantly from the values at P10 ($p < 0.01$). **c** The percentage of clusters that were located on spines was significantly affected by age, with a lower percentage located on spines at P10 compared to P20. The large variability observed at P10 is caused by the low number of gephyrin clusters per cell at this age point. **d** The percentage of spines that contained a gephyrin cluster was significantly higher at P20 compared to P10 and to P30. **c, d**: Values are given as the median $\pm$ the confidence interval. * $p < 0.05$; ** $p < 0.01$)

these excitatory dendritic shaft synapses can be commonly detected in non-pyramidal neurons, they constitute less than 2% of all excitatory synapses in CA1 principal cells (Megias et al. 2011). Nevertheless, the extent of these excitatory shaft synapses can exceed 10% in some specific subdomains of the dendritic tree, especially on thin distal dendritic segments, leading thereby to a potential bias of excitatory synapse counts (Megias et al. 2011).

In our analysis, we focused on the rat medial prefrontal cortex that constitutes the major portion of the medial wall of the hemisphere anterior and dorsal to the genu of the corpus callosum. This cortical region is implicated in attentional processes, working memory as well as behavioural

flexibility and is considered as the functional equivalent of the human prefrontal cortex (Uylings et al. 2003). Using dendritic spines as anatomical proxies to depict excitatory synapses, we have previously characterized the developmental pattern of excitatory synaptogenesis on pyramidal neurons in this region (Briner et al. 2011; Puskarjov et al. 2017). Here, we pursued these investigations by extending the analysis to simultaneous detection of excitatory and inhibitory synaptic inputs on the basal dendritic tree of the layer 2/3 pyramidal neurons. The reason behind this choice was mostly of practical nature since we have previously found that the basal dendritic tree has less intercellular variability in their thickness and branching patterns than the apical tree

which can either have a long, thick trunk or it can bifurcate early, giving rise to thinner branches (Briner et al. 2011). This small morphological variability of the basal dendritic arbor allows us to average cluster densities as a function of distance from the soma.

The gephyrin cluster densities observed with our approach are comparable to those reported in previous studies using either electron microscopy (Megías et al. 2001) or in utero electroporation of fluorescently-tagged gephyrin (Chen et al. 2012). Our data is also in line with earlier observations regarding the proportion of inhibitory terminals targeting dendritic spines (Beaulieu et al. 1992; Chen et al. 2012; Jasinska et al. 2010; Kubota et al. 2007). Furthermore, the mean diameter of gephyrin clusters in our analyses (for all age groups between 0.44 and 0.46 μm for gephyrin clusters on the shaft and 0.36 and 0.41 for clusters on spines) corresponds well to the existing literature on the size of synaptic contacts. In the mouse somatosensory cortex, the mean synaptic length of symmetric synapses was measured from electron microscopic images to be 0.24 μm (De Felipe et al. 1997). Slightly larger values have been reported using fluorescent labelling [Flores et al. 2015: mean cluster area 0.3 μm^2 , corresponding to a diameter of 0.6 μm (Flores et al. 2015)], which may be related to the spreading of fluorescent signals, as predicted by the point spread function. Immunostaining in brain slices against endogenous gephyrin or synaptically located GABAAR subunits, such as $\gamma 2$, also reveals fluorescent clusters of similar size (Studer et al. 2006). Furthermore, the nearly perfect colocalization between fluorescent TomGeph clusters and the presynaptic GABAergic marker VGAT supports the conclusion that the electroporated gephyrin incorporates into GABAergic synapses, and that the clusters detected in our analysis can be considered synaptic. In line with these results, others have also shown using electron microscopy that upon overexpression the fluorescent gephyrin clusters indeed correspond to inhibitory synapses (Chen et al. 2012; Flores et al. 2015).

We chose to use manual analysis, and to define the clusters as dense accumulations of several pixels that were several fold brighter than the background intensity in the surrounding dendrite, and that could be observed at least on two consecutive z levels (see “Materials and methods”). Since the z direction in confocal image stacks has a much lower resolution than the plane of the image, and spines projecting up or down from the dendritic shaft can usually not be detected. Consequently, gephyrin clusters located on z projecting spines have probably been classified as shaft clusters. It is also possible that due to the resolution limits of confocal microscopy, some small clusters located close to each other may have been interpreted as one big cluster. On the other hand, large gephyrin clusters corresponding on ultrastructural level to several synaptic contacts with complex forms (Flores et al. 2015), have probably in our

quantification been counted as a single cluster. Nevertheless, these large aggregates represent a very small percentage of all our analysed gephyrin clusters, and the error is thus negligible. Some studies also indicate the existence of non-synaptic nanoclusters (Specht et al. 2013), some of which may represent trafficking vesicles (Maas et al. 2006). However, these nanoclusters are much smaller than the synaptic gephyrin clusters and have an area of less than 0.01 μm^2 (Specht et al. 2013), which with our resolution would be interpreted as background. One can also argue that the overexpression of gephyrin might induce an increase in the cluster formation. However, gephyrin clustering is tightly regulated by numerous types of post-translational modifications, and thus the overexpression would only be expected to increase the soluble intracellular gephyrin reserve (Tyagarajan and Fritschy 2014). It has also been shown that the overexpressed gephyrin replaces the endogenous gephyrin molecules in synaptic clusters in a dose-dependent manner, while the total number of gephyrin molecules within the cluster remains remarkably constant (Specht et al. 2013). Because the level of overexpression in a given cell depends on the number of plasmids it received, a parameter that cannot be controlled during IUE, only cells with a strong plasmid expression were selected for our analysis. In this context, we have also provided evidence that IUE of gephyrin into precursors does not interfere with normal morphological development of pyramidal neurons since both dendritic arbour architecture and spine densities were comparable between electroporated and neighbouring non-electroporated cells. These results, together with our previous observations showing that the IUE procedure per se does not interfere with neuronal dendritic arbor (Fiumelli et al. 2013), thus strongly suggest the specificity of our approach.

Both the immunostaining against endogenous gephyrin and the analysis of fluorescent gephyrin clusters on single L2/3 pyramidal neurons revealed a steep increase in cluster density in the rat mPFC during the first postnatal weeks. Our results from the anti-gephyrin immunostaining are well in agreement with the previous electron microscopic studies analysing the volumetric density of GABAergic synapses (De Felipe et al. 1997; Micheva and Beaulieu 1996). In L2/3 and L5/6, the density of gephyrin immunopositive clusters roughly doubled first from P5 to P10, and again from P10 to P15, after which the density started to saturate towards the values measured at P30. During postnatal development, L1 was found to be most densely stained by gephyrin clusters. At P5, the cluster density in L1 was several fold higher than in the other layers at that age, and it also stayed higher at all later developmental stages studied. Since we did not specifically focus on neuropil, the difference in the volume taken up by cell bodies in each layer probably has an impact on the synaptic density. This can most strikingly be observed comparing L1 and L2/3. In neocortex, the L1 is nearly devoid of

cell bodies, containing only certain types of interneurons, whereas the L2/3 is characterized by the densely packed somata of pyramidal neurons (Shepherd and Grillner 2010). However, the difference between L1 and L2/3 was also previously reported for the density of GABAergic synapses per area of neuropil in electronmicroscopic images of mouse somatosensory cortex (De Felipe et al. 1997). Furthermore, in acute cortical brain slices from P0–7 rats, specific electrical stimulation of axonal fibers in L1 induced strong GABAergic excitation in pyramidal neurons in L2–6, highlighting the early importance of L1 in GABAergic signalling (Dammerman et al. 2000).

At the single cell level, we also observed a several fold increase in the density of GABAergic inputs between P10 and P20. This is well in agreement with our volumetric analysis done using the anti-gephyrin immunostaining, and highlights the rapid establishment of network connectivity during the first postnatal weeks. In the rat mPFC, the layer 2/3 pyramidal neurons are still relatively immature at P10, having thin and short dendrites with only few spines. Our results show that the gephyrin cluster density is also extremely low at this stage, suggesting that both glutamatergic and GABAergic connections are only starting to be formed. Furthermore, we show that at these early stages of network development, both types of synaptic contacts are distributed along the dendrites in a homogenous and seemingly random manner. However, by P15 a clear spatial pattern starts to emerge, and both dendritic spines as well as gephyrin clusters have a higher density in the more distal dendritic compartments. The temporal course of these events correlate with a time window in which several profound functional changes take place in the rat mPFC. In the EEG recordings, an abrupt switch from discontinuous bursting to adult-like continuous oscillations is seen around P10–11 (Brockmann et al. 2011), suggesting a substantial reorganization in the neuronal networks generating the patterns of activity. Indeed, the whole local inhibitory network has been reported to undergo rapid maturation in the mouse mPFC during the second postnatal week (Yang et al. 2014). During this time, the passive and active membrane properties of fast-spiking interneurons, as well as their electrical and synaptic coupling within the network develop rapidly, giving rise to sharper synaptic currents and higher firing rates (Yang et al. 2014). Furthermore, in light of the emerging concept suggesting that distal rather than proximal inhibition effectively dampens neuronal excitation (Gidon and Segev 2012; Rieubland et al. 2012), it is plausible that the spatial organization of synaptic inputs along the pyramidal dendrites, the pattern which in our data emerges between P10 and P15, is involved in the development of spatially and temporally precise processing of excitatory synaptic inputs.

At all developmental stages studied, we could identify gephyrin clusters located on dendritic spines. Dually

innervated spines were first described in the visual cortex in 1969 (Jones and Powell 1969), and they have later be shown to be preferentially targeted by thalamocortical afferents (Kubota et al. 2007). Interestingly, we could see a transient increase in the percentage of potentially double innervated spines at P20. Considering that GABAergic inhibition on dendritic spines can have highly localized effects on glutamatergic signalling (Chiu et al. 2013), it remains to be elucidated whether the increased inhibition within spines at P20 could have a role in refining of the circuitry. In adults, gephyrin clusters have been shown to have a high level of in vivo turnover in response to experience (Chen et al. 2012; van Versendaal et al. 2012; Villa et al. 2016), with the clusters on spines being the most dynamic. Thus, it is likely that the network connectivity will continue to be refined even after the adult densities have been reached.

In conclusion, we provide here an ex vivo method to simultaneously detect the spatial patterns of excitatory and inhibitory synaptic inputs onto pyramidal neurons at the single cell level. Using this approach, we describe for the first time the developmental pattern of synaptogenesis giving rise to fast excitation and inhibition on the basal dendritic tree of layer 2/3 pyramidal neurons in the rat medial prefrontal cortex. Given the importance of the spatial arrangement of synaptic inputs at the single cell level in determining neuronal excitability, the method described in this work may allow a better understanding of how inhibition settles together with excitation, and serve as basics for further modelling studies focusing on the geometry of dendritic inhibition during development (Gidon and Segev 2012). It may also be of particular interest in the context of pathologies such as neuropsychiatric disorders where alterations in neuronal circuits are observed at early developmental stages (Gonzalez-Burgos et al. 2010; McGlashan and Hoffman 2000; Penzes et al. 2011; Take-sian and Hensch 2013).

Acknowledgements We thank Michèle Brunet for excellent technical assistance, Kai Kaila, Jean-Marc Fritschy and Jozsef Kiss for instructive comments on the manuscript. This study was funded by the Swiss National Science Foundation, Berne, Switzerland, Grant 31003A-130625 (to LV).

Author contributions MAV, MDR, MK, ST, JMF, and LV designed the experiments. MAV, MDR, CML, HF and LV performed and analyzed the experiments. MAV, MDR and LV wrote the manuscript with input from the co-authors.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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