Pre-grid cell development of the immature medial entorhinal cortex

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Abstract

Grid cells in medial entorhinal cortex (MEC) code spatial aspects of a rodent’s environment from the third postnatal week onwards. Grid cells, the majority of which are stellate cells, are hypothesized to develop from topographic modules of synchronized neurons in superficial MEC. However, early MEC network activity prior to grid cell formation is largely unknown. Here, we show in vivo and in vitro that developing MEC neuronal networks in the second postnatal week were synchronously active in spatially-grouped modules of stellate cells and other neurons. These networks desynchronized just prior to the reported emergence of grid cell firing. Synchrony depended on MEC circuits. It was modulated but not driven by the immature hippocampus and was tightly-coupled to neighboring neocortical networks. Unlike mature MEC, developing modules were dominated by glutamatergic excitation rather than GABAergic inhibition. Our results demonstrate that intrinsically synchronous modules exist in immature MEC: these could play a key role in establishing circuitry necessary for adult grid firing.
Introduction

The mature medial entorhinal cortex (MEC) contains neurons that fire in a hexagonal pattern forming a neural representation of space (Hafting et al., 2005; Moser and Moser, 2008). These grid cells, the majority of which are stellate cells (Domnisoru et al., 2013; Schmidt-Hieber and Hausser, 2013), emerge during the third postnatal week in rodents (Langston et al., 2010). In young adults, superficial MEC layers are modulated by recurrent inhibition (Couey et al., 2013; Pastoll et al., 2013) and grid cell firing patterns arise from local inhibitory connectivity in attractor network models (Couey et al., 2013; McNaughton et al., 2006; Pastoll et al., 2013). Topographic activity modules are hypothesized to exist in immature MEC and form the basis for grid cell firing in the adult (McNaughton et al., 2006). However, it is not known how the network forms prior to spatial exploration and eye-opening.

In the adult, MEC is reciprocally connected to neocortex and parahippocampal regions, including the hippocampal formation (van Strien et al., 2009). Grid cell activity in MEC is shaped by excitatory hippocampal projections in the mature brain (Bonnevie et al., 2013) and CA1 place cell fields are less precise following MEC layer III ablation (Brun et al., 2008). Thus, in mature rodents recurrent connections exist between activity in MEC and neighboring hippocampal and neocortical regions. Immature hippocampus and cortex are characterized by synchronized patterns of network activity that are restricted to specific developmental periods (Blankenship and Feller, 2010) and essential for structural and functional maturation including axonal growth, synapse plasticity and sensory map formation (Blankenship and Feller, 2010; Spitzer, 2006). Therefore, we asked whether synchronous activity exists in MEC prior to eye-opening and exploration and tested the hypotheses that synchrony is driven by hippocampal inputs and mediated via GABAergic mechanisms, similar to immature hippocampus.

Using in vivo extracellular recordings, multiphoton calcium imaging, multi-electrode and paired cell recordings, we determined that synchronous modules are intrinsic to the early development of MEC networks in the rodent during a restricted developmental period prior to eye-opening (P13) and spatial navigation. In contrast to mature networks (Bonnevie et al., 2013; Couey et al., 2013; Pastoll et al., 2013), our data reveal highly synchronous glutamatergic networks in vivo and in vitro that are intrinsically-generated across layers. Immature MEC networks synchronize and modulate neighboring neocortex validating previous speculation (Garaschuk et al., 2000) but are neither driven by hippocampal nor neocortical activity. These modular networks, predicted by a computational model for path integration (McNaughton et al., 2006), may establish and tune the MEC circuitry to allow abrupt appearance of grid cells firing after eye opening (Wills et al., 2012).
Methods

Animal usage

All *in vivo* experiments were performed in compliance with the German laws and the guidelines of the European Community for the use of animals in research and were approved by the local ethical committee. Pregnant Wistar rats were obtained at 14-17 days of gestation from the animal facility of the University Medical Center Hamburg-Eppendorf, housed individually in breeding cages with a 12 h light/12 h dark cycle and fed ad libitum. All *in vitro* procedures involving animals were conducted in accordance with Dutch regulations and were approved by the animal ethics committee (DEC) of the VU University Amsterdam. C57BL/6 mouse pups of both sexes aged from postnatal day 7 (P7) to P15 were used for slice experiments.

Surgical procedure for *in vivo* recordings

Extracellular recordings were performed in the MEC (55 mm posterior to bregma and 7.5 mm from the midline) using experimental protocols as described previously (Hanganu et al., 2006; Brockmann et al., 2011). Under light urethane-anesthesia (0.125-1 g/kg; Sigma-Aldrich), the head of the pup was fixed in the stereotaxic apparatus (Stoelting, Wood Dale, IL) using two metal tubes cemented on to the nasal and occipital bones, respectively. The bone over the MEC was carefully removed by drilling holes of less than 0.5 mm in diameter. Removal of the underlying dura mater by drilling was avoided, since leakage of cerebrospinal fluid or blood can dampen the cortical activity and single neuronal firing (I. Hanganu-Opatz, personal observations). The body of the animals was surrounded by cotton wool and kept at a constant temperature of 37 °C by placing it on a heating blanket. During recordings, urethane anesthesia (0.1-0.2 times the initial dose) was given when the pups showed any sign of distress. After a 20-60 min recovery period, multielectrode arrays (Silicon Michigan probes, NeuroNexus Technologies) were inserted at 10° from the vertical plane into MEC at a depth of 45 mm. The electrodes were labeled with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine, Invitrogen) to enable post-mortem reconstruction of electrode tracks in the MEC in histological sections (Figure 4.1A). One or two silver wires were inserted into the cerebellum and served as ground and reference electrodes. Miniature earphones placed under the pup’s body were sensitive enough to detect the smallest visible movements of the limbs as well as the breathing of pups during recordings. The sleep-like conditions mimicked by the urethane anesthesia (Clement et al., 2008) had no major effect on the properties and dynamics of early network oscillations (Yang et al., 2009).

Protocols for *in vivo* recordings

Simultaneous recordings of LFP and MUA were performed from the MEC using one-shank 16-channel Michigan electrodes (0.5-3 MΩ). The recording sites were separated by 50 μm.
The recording sites covered the MEC and lateral entorhinal cortex. Both LFP and MUA were recorded for at least 900 s at a sampling rate of 32 kHz using a multi-channel extracellular amplifier (Digital Lynx 4S with no gain, Neuralynx, Bozeman, MO) and the corresponding acquisition software (Cheetah). During recording the signal was band-pass filtered between 0.1 Hz and 5 kHz (Digital Lynx).

Confirmation of recording locations
DiI (1,1’-dioctadecyl-3,3,3’,3’-tetramethyl indocarbocyanine, Invitrogen) was used for marking electrode recording location in vivo. After recording, the pups were deeply anesthetized with 10 % ketamine (ani-Medica, Senden-Bögensell, Germany) and 2 % xylazine (WDT, Garbsen, Germany) in NaCl (10 ml/g body weight) and perfused transcardially with 4 % paraformaldehyde dissolved in 0.1M phosphate buffer, pH 7.4. The brains were removed and postfixed in the same solution for at least 24 h. Blocks of tissue containing the MEC were sectioned in the coronal plane at 100 µm and air dried. Fluorescent Nissl staining was performed using the NeuroTrace 500/525 green fluorescent Nissl stain (Invitrogen). Briefly, rehydrated slices were incubated for 20 min with 1:100 diluted NeuroTrace. Sections were washed, coverslipped with Fluoromount and examined using the green filter (AF 488).

Analysis of in vivo electrophysiology data
Data were imported and analyzed off-line using custom-written tools in MATLAB (Mathworks) software. To detect oscillatory events, the raw data were filtered between 4 and 100 Hz using a Butterworth 3-order filter. Only discontinuous events lasting >100 ms, containing at least 3 cycles, and being not correlated with movements (twitches) were considered for analysis. The discontinuous theta bursts in the MEC were analyzed in their occurrence (defined in Hz), duration, max amplitude (defined as the voltage difference between the maximal positive and negative peaks), and dominant frequency. Time-frequency plots were calculated by transforming the LFP using Morlet continuous wavelet. Minimal and maximal intensities in power were normalized to values between 0 and 1 and were displayed in dark blue and red, respectively.

Preparation of horizontal brain slices
Horizontal entorhinal cortex slices were prepared as described previously (Dawitz et al., 2011). Briefly, animals were rapidly decapitated and their brains dissected out under ice cold cutting solution containing (in mM): 110 Choline chloride, 26 NaHCO₃, 10 D-glucose, 11.6 sodium ascorbate, 7 MgCl₂, 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH₂PO₄ (Merck), and 0.5 CaCl₂. 300 µm thick slices were obtained using a HR2 slicer (Slicer HR-2; Sigmann Elektronik, Hueffenhardt, Germany; vibration frequency: 36 Hz, vibration amplitude: 0.7 mm, propagation speed: 0.05 mm/s). After a minimum recovery period of one hour slices were transferred into a holding chamber containing artificial cerebral spinal fluid (aCSF) with
slightly elevated magnesium levels at room temperature composed of (in mM): 125 NaCl, 26 NaHCO$_3$, 10 D-glucose, 3 KCl, 2.5 MgCl$_2$, 1.6 CaCl$_2$, and 1.25 NaH$_2$PO$_4$ (Merck) and continuously bubbled with carbogen gas (95 % O$_2$, 5 % CO$_2$).

**Reagents**

If not indicated otherwise, all reagents were purchased from Sigma-Aldrich. For pharmacology the following concentrations of blockers were added to the standard aCSF: DL-2-Amino-5-phosphonopentanoic acid (DL-APV, Abcam): 100 µM, 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, Abcam): 2 µM, Kynurenic Acid (KynA): 2 mM, Gabazine (Tocris): 10 µM (blockade of phasic and tonic), CGP 55845 (CGP, Tocris): 4 µM, Carbenoxolone (CNX, Tocris): 100 µM, and Glycyrrhizic acid (GZA, Abcam): 100 µM. Low and high calcium concentrations were achieved by using 0.6 mM or 2.6 mM CaCl$_2$ for the standard aCSF.

**Fura-2 AM bulk loading and two-photon data acquisition**

After recovery slices were transferred into an interface-chamber filled with 1 ml elevated magnesium aCSF heated to approximately 34 ºC. 50 µg Fura-2 AM (Invitrogen) diluted in 9 µl DMSO and 1 µl Pluronic F-127 (20 % solution in DMSO; Invitrogen) was pipetted directly on top of the entorhinal cortex and incubated for 20 minutes (for slices of P7/8 animals) to 40 minutes (for slices of P14/15). Slices from animals older than P12 were preincubated for three minutes in 3 ml aCSF with 8 µl 0.5 % cremophor (Fluka) diluted in DMSO to facilitate Fura-2 AM uptake. After incubation slices were briefly transferred back into the holding chamber and any residual surface dye was washed off.

To improve stability of recordings a poly(ethyleneimine)-solution (1ml poly(ethyleneimine) in 250 ml boric buffer containing 40 mM boric acid and 10 mM sodium tetraborate decahydrate) was used to attach slices onto the recording chambers. After coating of the recording chambers for one hour slices were mounted in elevated magnesium aCSF. The attached slices were placed into a humidified interface container perfused with carbogen and left for at least one hour to achieve a stable attachment and allow for esterase activity to take place within the neurons to trap and activate Fura-2 AM.

Functional multiphoton calcium dye network imaging data was acquired on a Trimscope (LaVision Biotec) connected to an Olympus microscope using a Ti-sapphire (Coherent) laser tuned to 820 nm wavelength. All recordings of sMEC were acquired using a 20x lens (NA 0.95) and a 350x350 µm field of view. Parallel recordings of dMEC and sMEC were obtained with a 10x objective (NA 0.3) and a 700x700 µm field of view. During data acquisition slices were continuously perfused with oxygenated standard aCSF (recipe as above but 1.5 mM MgCl$_2$) heated to approximately 27 ºC. Superficial layers of the medial entorhinal cortex were visually identified using light microscopy. Using a Hamamatsu C9100 EM-
CCD camera as a detector, two time-lapse movies (2000 frames each) in sMEC or sMEC and dMEC were acquired with a sampling frequency of approximately 10 Hz. For neuron detection, at the end of each recording condition a z-stack ±20 µm around the central plane with 1 µm slice thickness was acquired.

For simultaneous network imaging and cell-attached stellate cell recording, data was acquired on a Leica RS2 two-photon laser-scanning microscope tuned to 820 nm wavelength with a 20x lens and 400x400 µm field of view. Time-lapse data was acquired on a PMT at a rate of 565 msec/frame (425 frames each) in sMEC with simultaneous cell-attached recording. Spike rates were acquired using Clampex 10.2 (MDS Analytical Technologies) at an acquisition rate of 10 kHz. Cell identity was electrophysiologically determined with a step profile following breakthrough (see Patch-clamp recordings below). Identified stellate cells in layer II were filled with Alexa 594 (80 µM, Invitrogen) for additional anatomical identification.

Drugs were washed in for minimally 10 minutes prior to recording except for CNX and GZA that were incubated for at least 30 minutes. Calcium concentrations were changed at least 20 minutes prior to recording onset. Hippocampal and NeoC lesions as well as mini-slices were prepared under a low magnification microscope (4x) using a surgical knife. After lesioning two time-lapse movies and a z-stack were recorded as described above.

**Analysis of imaging data**

Custom-built MATLAB (Mathworks) software was used to analyze calcium data (Hjorth et al., 2015). Neurons were detected using the z-stack recorded at the end of each condition. After localizing putative neuronal centers using local intensity peaks, deformed spheres were placed around these centers within the 3D stack to fit the putative neurons. A neuron was added to the contour mask if the spheres had the volume corresponding to a radius of 2-20 µm and the intersection of the sphere and the imaging plane had a minimum area of 25 µm². The pia was indicated on the final mask to determine the distance to pia of individual neurons in the slice. For analysis of spatial distributions in superficial networks, only neurons located between 50 and 300 µm from the pia were analyzed due to sparse cell density in layers I and IV (Figure 4.2B, gray bars). For low resolution recordings, neurons with 100 to 500 µm distance to pia were analyzed.

For each detected neuron the corresponding fluorescent raw trace was extracted from the two consecutive time-lapse recordings and the relative fluorescence trace was calculated (ΔF/F). The baseline was estimated using the running median of the relative trace. Frames with a drop in intensity of at least 10 % in the relative trace were considered a putative event. Additionally the event had to be at least 1 SD below the baseline and the trace had to
remain significantly below this baseline for a minimum of 5 frames (tested with a one-sided t-test). To confirm onset times and to increase sensitivity of onset detection we repeated this procedure three times excluding the detected putative events from the running median and using a two sided t-test comparing the putative event frames against the filtered baseline for significance in the consecutive iterations. The result was manually inspected and where necessary, corrected.

Groups of synchronized neurons were detected as follows: Onset times of detected events from all neurons were summed together on a frame-by-frame basis and the resulting activity vector was smoothed using a Gaussian. All local peaks exceeding the threshold were defined as network events. The threshold was 5 times the SD of 500 activity vectors derived from the same traces but with randomly shuffled inter-event intervals. A neuron was assigned to the synchronized group if the network events smoothed with a Gaussian overlapped for at least 40% with the total number of event onsets in an individual neuron smoothed with the same Gaussian.

Thus, from this analysis three categories of neurons were derived: silent neurons that do not show any activity, active neurons that show activity and synchronized neurons that are active and whose activity is synchronized to other neurons in the network. The frequency of each group and the network event frequency were calculated. Finally, another MATLAB (Mathworks) script was used to align masks of different experimental conditions to extract repeated measurements of parameters for testing based on individual neurons. All frequency values in the text and figures are derived from these within neuron comparisons and are thus analyzed on single neuron bases, except for the mini-slice experiments (Figure 4.5) where re-alignment of individual neurons was impossible. Here, the average frequencies of all neurons per slice were used. To assess the spatial spread of synchronized neurons within the field of view the average distribution of synchronous neurons around a synchronous neuron was calculated, and compared to the average distribution of neighboring asynchronous neurons. As an additional test, the synchronous neuron distribution was also compared to a shuffled control.

Synchronized activity is referred to as network events to clarify activity measures taken from calcium imaging data. Similar rhythmic activity measured in vivo and in vitro with field and patch-clamp recordings from individual cells are referred to as network bursts throughout the manuscript.

**Field recordings and analysis**

dMEC, sMEC, NeoC (perirhinal cortex) and the different hippocampal areas were visually identified with an Olympus microscope (4x lens) using oblique contrast. Field electrodes (chloride-coated silver electrodes inserted into 2-3 MΩ borosilicate glass pipettes filled with
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aCSF) were lowered into the regions of interest using micromanipulators (Luigs-Neuman, Ratingen) and placed at regions with good signal:noise readings. Field potentials were acquired with Multiclamp 400B amplifiers (Molecular Devices) using Clampex 10.2 (MDS Analytical Technologies) at an acquisition rate of 10 kHz.

Analysis was carried out using custom-made MATLAB (Mathworks) software. First, signals were high-pass filtered at 1 Hz to eliminate any slow frequency drift of the signal. Then, a threshold of 5.5 times the standard deviation (SD) was applied to detect field potentials. For the dMEC-sMEC recordings customized thresholds (between 4.5 and 12 times SD) were used to achieve the optimal detection rates. Spikes were grouped as one event if they were preceded by another spike within 3 seconds. The first spike of an event was defined as the event onset and used to calculate event frequency. Given the long duration of network bursts, a synchronous event between different regions and sMEC was defined as the occurrence of an event in the region of interest within a time-window of 2.5 s preceding or following an sMEC event. The proportion of synchrony between different regions relative to sMEC was then calculated.

**Multi-electrode array recordings and analysis**

Slices were mounted (see bulk-loading calcium dye methods above) on planar 100/10-ITO-PR multi-electrode arrays (MEAs) consisting of 60 recording electrodes spaced 100 μm (Multi Channel Systems, GmbH) with the MEC region positioned on top of the electrode grid (Suppl. Figure 4.4).

Spontaneous activity and local field potentials were measured while the recording chamber was constantly bathed with standard aCSF at a flow rate of 4 ml/min and kept at 30±0.5 °C. Data acquisition was performed at 5 kHz with MCRack software (Multi Channel Systems). Extracellular stimulation was applied with a silver electrode inserted into a glass borosilicate pipette filled with standard aCSF.

Data was analyzed with custom-made MATLAB (Mathworks) software according to the anatomical layers of MEC. Evoked excitatory post-synaptic potentials were averaged over 20 repetitions and their amplitude was analyzed. For analysis of the occurrence of network burst events prior to and after stimulation, the optimal electrode recordings with sufficient signal to noise ratio to unambiguously detect network burst events were selected in a slice and network burst events up to 1 s prior to and 1 s after stimulation were quantified.

**Patch-clamp recordings**

For patch-clamp recordings the same recording apparatus was used as for field recordings (see above) but the microscope was equipped with a 60x lens. Borosilicate pipettes with a resistance of ~10 MΩ were filled with intracellular solution containing (in mM): 148 K-glu-
conate, 1 KCl, 10 Hepes, 4 Mg-ATP, 4 K_2-phosphocreatine, 0.4 GTP and 0.2 % biocytin, adjusted with KOH to pH 7.3. For cell-attached recordings (2-10 min) active cells of interest were selected in dMEC and sMEC and recorded simultaneously in GΩ-resistance seal configurations. After up to 10 min continuous voltage-clamp recordings, whole-cell access was made if possible and step protocols were acquired in whole-cell Current-clamp mode to electrophysiologically-identify neuron type and to fill neurons with biocytin for post-hoc anatomical confirmation. To measure synaptic inputs of identified neurons in some cases up to 10 min Voltage-Clamp, whole cell recordings were acquired. After recordings, slices were fixed in 4 % Paraformaldehyde for at least one week before staining.

Cell-attached data was analyzed using MATLAB (Mathworks) scripts adapted from the field recording analysis. Due to stable baselines, no filtering was applied. Customized thresholds for action-potential (AP) detection ranged from 2-40 times SD. APs were grouped to be part of one network burst if they were preceded by another spike within a timewindow of 1.5 s. As a measure of synchrony, the proportion of events in sMEC that are followed or preceded within 1 s from time of event onset in the dMEC was calculated.

For anatomical identification slices were stained for biocytin using a modified avidin-biotin-peroxidase method. Note that staining of the entire dendritic tree was limited due to the high input resistance due to the 10 MΩ pipettes.

Statistics

For in vivo recordings, data in the text are presented as mean ± SEM. Statistical tests used are indicated in the figure legends for individual experiments. In general, to compare two conditions within the same experiment a paired t-test was used. To compare means from different experiments (i.e. the synchrony or the different ages where GABA-Rs were tested) independent t-tests were performed. Changes upon GABA-R blockade and timelags were assessed based on one-sample t-tests against a hypothetical mean of 0. The difference in mean distance between synchronized to synchronized and synchronized to other neighbors was tested with a Wilcoxon Signed Rank test. The distributions of synchronized to synchronized neighbors and synchronized to non-synchronized neighbors were compared using a Kolmogorov-Smirnov test.

Multiple means were compared using one-way ANOVAs. Post-hoc analysis was carried out using the Bonferroni t-test. Differences between distributions were assessed using the Kolmogorov-Smirnov test (Figure 4.2, spatial distributions data). Categorical data (failures and bursts upon stimulation) were analyzed using chi square statistics.

Reported is the abbreviation of test statistic with the degrees of freedom in brackets equaling the value of the test statistic. Differences were defined as significant if p-values were smaller than 0.05. A * indicates significance (p<0.05), a # indicates a trend (p<0.01).
Results

Spontaneous bursts of activity in developing medial entorhinal cortex

Discontinuous oscillatory activity is a hallmark pattern during early network development of many brain regions (Blankenship and Feller, 2010). To determine whether MEC exhibits such rhythmic network activity, we performed extracellular recordings of the local field potential (LFP) and multiple unit activity (MUA) in neonatal [postnatal day (P) 7-8] urethane-anesthetized rodent pups (Figure 4.1A, n=4 pups). Neonatal MEC showed discontinuous patterns of oscillatory activity with a relatively high occurrence (P7-8: 0.12±0.01 Hz, mean±SEM, Figure 4.1B,D) when compared to neocortical regions (Yang et al., 2009). Oscillatory bursts were of short duration and large amplitude (3.3±0.2 s, 249±7.3 µV, n=160 events) and contained a predominant theta band that was phase-locked to high-frequency MUA (Figure 4.1C, 6.2±0.1 Hz, n=160 events). Higher frequency components in the gamma band range could be additionally detected.

Figure 4.1: Patterns of network activity in the neonatal MEC in vivo. (A) Digital photomontage reconstructing the location of the DiI-labeled recording electrode (orange) in the MEC of a Nissl-stained 100 µm-thick coronal section (green) from a P7 pup. Yellow dots mark the recording sites covering the entorhinal layer II/III. Scalebar 1 mm. (B) Band-pass filtered (4-100 Hz) extracellular field potential recording of discontinuous oscillatory activity in the MEC accompanied by the color-coded wavelet spectra of the LFP at identical time scale. The red dotted line marks the lower border of gamma frequency band (30 Hz). (C) Characteristic theta burst displayed before (top) and after band-pass (4-30 Hz) filtering (middle) and the corresponding MUA after 200 Hz high-pass filtering (bottom). Color-coded frequency plot shows the wavelet spectrum of LFP at identical time scale. (D) Occurrence of MEC theta bursts (mean ± SEM) with superimposed values for individual animals (n=4).
To unravel the mechanisms underlying the network bursts we utilized an in vitro slice approach. Field recordings in horizontal slices of sMEC confirmed the occurrence of network bursts with activity patterns similar to in vivo (unpaired t-test, \( t(25)=-1.5, p=0.15 \) Figure 4.1D, Suppl. Figure 4.1C). Using multiphoton calcium imaging for single cell resolution we found that networks were comprised of silent and active cells, of which the majority was synchronously active (Figure 4.2A). To establish the developmental time-course of activity we mapped spontaneous network events during the second postnatal week, prior to grid cell onset (Langston et al., 2010). In each neuron somatic calcium events reflecting suprathreshold activity were binarized and their synchronization across the network was calculated (Figure 4.2B-D, see Methods). Network activity peaked at P10/11 with the highest proportion of active neurons (40.1±4.9 % cells) and significantly decreased towards P14/15 (7±2 % cells, Figure 4.2C, blue bars). Within the active network, the fraction of active neurons that were synchronized followed a similar profile, peaking at P10/11 (31±4 % cells) and decreasing by P14/15 (2±1 % cells, Figure 4.2C, black bars). The frequency of active neurons was strongly developmentally regulated being higher at P10/11 (0.055±0.007 Hz) than at P14/15 (0.008±0.004 Hz). Frequency of synchronized bursts of events across the network (‘network events’ – see Methods for definition), also peaked at P10/11 (0.050±0.013 Hz) and virtually disappeared by P14/15 (0.002±0.001 Hz, Figure 4.2D). Despite a decrease in activity of all neurons with age, the decrease was more prominent for synchronized network events, changing significantly from comprising 79.3±11.1 % of all activity at P10/11 to 29.6±15.5 % at P14/15. Unless otherwise stated, all data in the following paragraphs is reported from peak activity ages P8-11.

Configuration of synchronous network modules

In adults, grid cells are the predominant cell type found in layer II sMEC (Sargolini et al., 2006) and of these, the majority are stellate cells (Domnisoru et al., 2013; Schmidt-Hieber and Hausser, 2013). Simultaneous cell-attached recordings of stellate cells with calcium imaging (Figure 4.3A left) confirmed that immature stellate cells fire in synchrony with the network and thus a main component of the developmental bursts observed in sMEC (Figure 4.3A, Suppl. Figure 4.1). To test whether synchronized neurons were not only functionally- but also spatially-clustered in modules, as proposed by a theoretical model for grid cell development (McNaughton et al., 2006), we investigated their physical distribution across sMEC (Figure 4.3B, Suppl. Figure 4.2). Synchronized stained neurons were homogenously distributed with similar density across both superficial layers (II and III, Figure 4.3B). However, the average distance between a synchronized neuron and its synchronized neighbors was significantly shorter compared to its non-synchronized neighbors (median: 156 µm synchronized vs. 196 µm non-synchronized, Figure 4.3C left, middle). Further investigation using Sholl-like analyses revealed a distance-dependent distribution profile with the greatest sMEC (Figure 4.3B, Suppl. Figure 4.2). Synchronized stained neurons were homogenously
Figure 4.2: Developmental dynamics of spontaneous sMEC activity in vitro. (A) Contour map of Fura-2 AM ester bulk-loaded cells in superficial medial entorhinal cortex networks imaged at P10/11. Active neurons (blue), silent neurons (red) and synchronized neurons (black) indicated. Scalebar 50 µm. Example traces of silent and synchronously active neurons. (B) Representative raster plots of sMEC network activity at P8/9, P10/11, and P14/15, color coded as in A. (C) Quantification of the fraction of active and synchronized neurons during postnatal development (n(networks imaged)=14, 7, 11, 10 respectively at P8/9, P10/11, P12/13 and P14/15, indicated in individual bars) showed a significant age-dependent peak in activity (Bonferroni corrected, P8/9 vs. P14/15, p<0.001, and P10/11 vs. both P12/13, p<0.03, and vs. P14/15, p<0.001, one-way ANOVA, F(3,41)=10.1) and synchronization (Bonferroni corrected, P8/9 vs. P14/15 p<0.01, P10/11 vs. P12/13 p=0.001 and P10/11 vs. P14/15, p<0.001, one-way ANOVA, F(3,41)=11.1) at P10/11. (D) Frequency of network events (see Methods for definition) is highest at P10/11 and virtually absent by P14/15 (Bonferroni corrected: P8/9 vs. P14/15, p<0.05, P10/11 vs. P12/13, p<0.05, and P10/11 vs. P14/15, p<0.01, one-way ANOVA, F(3,41)=5.8).
distributed with similar density across both superficial layers (II and III, Figure 4.3B). However, the average distance between a synchronized neuron and its synchronized neighbors was significantly shorter compared to its non-synchronized neighbors (median: 156 µm synchronized vs. 196 µm non-synchronized, Figure 4.3C left, middle). Further investigation using Sholl-like analyses revealed a distance-dependent distribution profile with the greatest number of synchronized neighbors grouped 50-100 µm away (mean 4.54) and those of non-synchronized neighbors at 150-200 µm (mean 10.93, Figure 4.3C right). A comparison of these spatial patterns revealed a significantly different, non-random distribution of synchronized compared to non-synchronized neighboring neurons within the stained networks (red line, Figure 4.3C right).

**Non-hippocampal drive of MEC synchrony**

To establish whether hippocampal connections to MEC exist *in vitro* during the second postnatal week, we evoked field excitatory postsynaptic potentials (fEPSPs) in sMEC by stimulation of dentate gyrus (DG) or CA1 in the immature (P8-P10) hippocampus and compared them with local stimulation in deep layers of MEC (dMEC, Figure 4.4B,C). Using a 60-electrode array in MEC (Figure 4.4A, left) we recorded high failure rates for both CA1- and DG-stimulation (76% and 57% of total experiments, respectively) compared with a total absence of failures upon dMEC stimulation (Figure 4.4C). Therefore, direct functional connections exist between the hippocampus and sMEC but they are highly unreliable and significantly weaker than local projections from dMEC layers.

To test whether spontaneous network activity in sMEC could be driven by the immature hippocampus we made simultaneous field recordings in CA1-sMEC, CA3-sMEC and CA1-CA3-sMEC (Figure 4.4A middle, Figure 4.4D). Frequency of spontaneous activity was significantly higher in sMEC compared to CA1 (0.1±0.02 Hz vs. 0.04±0.015 Hz, Figure 4.3E) and CA3 (0.09±0.015 Hz vs. 0.04±0.008 Hz, Figure 4.4E). Furthermore, the proportion of bursts that were synchronized with sMEC (Figure 4.4E, right), was significantly lower for CA1 (24±8%) and CA3 (18±5%) than the clear, consistent synchrony observed between sMEC-dMEC (90±5%) and sMEC-Neocortex (NeoC, 9±10%, Figure 4.5C).

To verify that CA1 and CA3 do not initiate MEC events, we quantified the occurrence of bursts of sMEC network activity one second before and after field stimulation in CA1 and DG (Figure 4.4A left, see Methods for ‘network burst’ definition). The majority of sweeps showed no evoked network burst within one second following stimulation in either CA1 (574/590 trials post-stim, Figure 4.4F, left) or DG (565/570 trials post-stim, Figure 4.4F, right). Furthermore, the low occurrence of network bursts upon stimulation did not differ from spontaneous burst rates observed up to one second prior to stimulation in CA1 (12 events= 2% trials pre-stim, 16 events= 3% trials post-stim) or DG (2 events <1% trials...
pre-stim, 5 events <1% trials post-stim), indicating that the hippocampus cannot reliably evoke the developmental network bursts in sMEC. Finally, to investigate the modulatory effect of the hippocampus proper upon sMEC network events, we recorded network events using calcium imaging before and after a hippocampal lesion (Figure 4.4A, right). Following removal of hippocampal inputs, the number of synchronized neurons increased significantly (27±6% to 39±6%, Figure 4.4G, left). However, the network event frequency remained stable (pre: 0.07±0.024, post: 0.07±0.018 Hz, Figure 4.4G, right) demonstrating that the hippocampus modulates but does not drive the synchronized spontaneous sMEC network activity.
Pre-grid cell development of the immature medial entorhinal cortex

Figure 4.4: Immature hippocampus does not drive synchronous network activity in MEC. (A) Left, Overview of MEA recordings (blue grid, MEC) and stimulation sites (Dentate gyrus (DG), CA1 and dMEC), middle, field recording sites (CA1, CA3, sMEC) and right, recording site of multiphoton network calcium imaging (red box) with hippocampal lesion (red line). (B) Example traces of mean evoked fEP-SPs of representative experiments (n=20 sweeps) upon dMEC, CA1 and DG stimulation recorded using a multielectrode array in sMEC. (C) Evoked fEPSP amplitudes (black) and response failures (gray) for dMEC (n=25 slices, 0 % failures), CA1 (n=26 slices, of which 76 % failed to evoke sMEC) and DG (n=25 slices, 57 % failures). Mean of successful responses was significantly bigger in sMEC than in CA1 (unpaired t-test, t(34)=4.00, p<0.001) and DG (unpaired t-test, t(29)=4.57, p<0.0001). (D) Example of typical...
Field recordings (1 Hz highpass filtered) show rhythmic burst activity in sMEC but less frequent, single spike activity in CA1 and CA3. (E) Left, Network event frequency is significantly lower in CA1 and CA3 compared with sMEC (paired t-tests, t(6)=6.95, p<0.001 and t(11)=6.12, p<0.0001 respectively). Right, low levels of synchrony exist between CA1-sMEC and CA3-sMEC that are significantly weaker than sMEC-dMEC and sMEC-NeoC (Unpaired t-tests, CA1-sMEC vs. NeoC-sMEC: t(15)=3.87, p<0.01, CA3-sMEC vs. NeoC-sMEC: t(20)=5.72, p<0.0001, CA1-sMEC vs. dMEC-sMEC: t(12)=6.67, p<0.0001 and CA3-sMEC vs. dMEC-sMEC: t(17)=9.24, p<0.0001) gray dashed lines, see Figures 5 and 6 respectively for comparative data.

(F) Evoked field stimulation in CA1 (left) and DG (right) failed to reliably evoke network bursts in sMEC and did not alter the rate of spontaneous network bursts (chi-square with Yates correction, CA1: chi-square(1)=0.33, p=0.56, DG: chi-square(1)=0.58, p=0.45). (G) Removal of hippocampus significantly increased the number of synchronized neurons (paired t-test, t(7)=3.09, p<0.05) but did not change the network frequency of synchronized events in sMEC (paired t-test, t(7)=1.23, p=0.26). N experimental numbers indicated on bars.

Neocortical synchrony is paced by intrinsic sMEC activity

Immature entorhinal cortex has been proposed as a cortical ‘pacemaker’ whose intrinsic activity drives NeoC during early postnatal development (Garaschuk et al., 2000; Namiki et al., 2013). To confirm that sMEC can self-generate its own network activity, we utilized calcium imaging in isolated MEC mini-slice preparations (Suppl. Figure 4.3A). Frequency of network events dropped upon isolation (0.10±0.02 Hz vs. 0.04±0.01 Hz, Suppl. Figure 4.3C, right). However, we observed no changes in fractions of active (47±3 % vs. 47±5 %, Figure 4.5B, left), synchronized neurons (31±4 % vs. 29±6 %, Suppl. Figure 4.3B, right) or activity levels (0.04±0.009 Hz vs. 0.02±0.006 Hz, Suppl. Figure 4.3C, left) indicating that intrinsic synchrony persisted, similar to the intact slice preparation. To test whether sMEC bursts drive neocortical activity, we then used paired field recordings in both sMEC (blue traces) and NeoC (gray traces) to measure spontaneous network bursts (Figure 4.5A,B top). NeoC network bursts were highly synchronized with sMEC bursts (Figure 4.5B, top, Figure 4.5C 79±30 %, ‘pre-cut’, 4.5D). We saw no significant time-lag between sMEC and NeoC to indicate a consistent origin of activity and propagation (230±218 ms, Figure 4.5D: example recording, 4.5E). To directly test if sMEC paces NeoC activity, we lesioned all interregional connections (Figure 4.5A, bottom). Both sMEC and NeoC displayed rhythmic network bursts following lesioning (Figure 4.5B, bottom), indicating that NeoC activity must be partly generated by a source other than sMEC. However, sMEC-NeoC synchrony was strongly decreased post-lesion (Figure 4.5C, pre: 79±10 %, post: 29±8 %, Figure 4.5D). NeoC network burst frequency significantly dropped following separation from sMEC (0.09±0.02 Hz vs. 0.06±0.02 Hz, Figure 4.5F, right). In contrast, no change in frequency of network bursts occurred in sMEC (0.07±0.02 Hz vs. 0.07±0.01 Hz, Figure 4.5F, left), suggesting that NeoC bursts synchronize to sMEC activity.

dMEC-sMEC time-locked network synchrony

Pyramidal neurons in dMEC connect to both principal neurons and interneurons in sMEC
Pre-grid cell development of the immature medial entorhinal cortex (Hamam et al., 2000; van Haeften et al., 2003). To assess whether dMEC network events synchronize with sMEC during the second postnatal week of development, we measured their activity simultaneously using low-magnification calcium imaging (Figure 4.6A). dMEC and sMEC layers were spatially-defined prior to event analysis (Figure 4.6A). Synchronized neurons occurred in both dMEC and sMEC layers (Figure 4.6B) with no difference in density between layers or relative to pial distance (Figure 4.6B). Network events were synchronized across individual neurons in deep and superficial layers (Figure 4.6C) with a similar rhythmic frequency (Figure 4.6D, 0.04±0.01 Hz vs. 0.03±0.01 Hz). For millisecond-level resolution, we investigated the temporal relationship between dMEC and sMEC using paired cell-attached recordings between sMEC stellate cells and dMEC pyramidal neurons and also field recordings (Figure 4.6E-H, Suppl. Figure 4.4A-C). Network bursts were highly synchronized (Figure 4.6G, 76±6 %, n=14 (cell-attached), Suppl. Figure 4.4B,C, 89±5 %,

Figure 4.5: Spontaneous network activity in immature MEC is synchronized with but not driven by neocortex. (A) Schematic view of field recordings and lesion site. (B) Example recordings of simultaneously pre- (top traces) and post-lesion (lower traces) sMEC (blue) and superficial neocortex (NeoC, gray). (C) Significant decrease in the proportion of synchronized events between sMEC and NeoC post-lesion (paired t-test, t(7)=5.10, p<0.01). Blue line indicates mean values, n=8 slices. (D) Representative time-lag histogram for onset times of spontaneous network activity between sMEC and NeoC pre- (black bars) and post-lesion (red open bars). All events with a timelag ±2.5 s (gray dotted lines) are considered synchronized. (E) No reliable time-lag (one-sample t-test, t(9)=1.06, p=0.32) indicating no directionality of activity between sMEC and NeoC. (F) No change in the frequency of sMEC network activity following lesion (paired t-test, t(7)=0.10, p=0.92) but significant decrease in NeoC network activity (paired t-test, t(7)=3.19, p<0.05). N experimental numbers indicated on bars.
n=7 (field recordings)). There was no significant time-lag between cell pairs or layers, providing no evidence that deep layers were driving spontaneous superficial network activity (Figure 4.6H, cell-attached median timelag: 12±20 ms, Suppl. Figure 4.4C field recordings median timelag: 772±1784 ms). Furthermore, paired whole-cell voltage-clamp recordings from identified stellate cells in sMEC and dMEC pyramidal neurons confirmed strikingly similar spontaneous input patterns during network bursts (Figure 4.6E,G, lower traces). In addition to spontaneous activity, field stimulation in dMEC failed to evoke network bursts in sMEC in 99% of trials (Suppl. Figure 4.4D). The low rate of induction (6/600) was not significantly different from the spontaneous network burst rate observed prior to stimulation (4/600). Similar results were obtained for dMEC-dMEC stimulation (Suppl. Figure 4.4D). Therefore, dMEC is unlikely to drive spontaneous synchronous network activity in superficial layers.
Glutamatergic activity underlies immature MEC synchrony

Synchronized network activity during the first postnatal week is dependent upon glutamatergic or GABAergic mechanisms in NeoC and hippocampus, respectively (Allene et al., 2008; Conhaim et al., 2011; Garaschuk et al., 1998; Garaschuk et al., 2000). Given the prominent GABAergic connectivity within adult sMEC (Couey et al., 2013; Pastoll et al., 2013) and that MEC is part of the parahippocampal region (van Strien et al., 2009), we hypothesized that spontaneous network activity would be mediated by GABAergic receptors. To rule out glutamatergic mechanisms, AMPA and NMDA receptors were selectively blocked (Figure 4.7A). Blockade of either receptor decreased overall frequency of activity (CNQX: 0.03±0.002 Hz vs. 0.02±0.003 Hz, DAPV: 0.06±0.005 Hz vs. 0.034±0.003 Hz, Figure 4.7B left). However, the number of synchronized neurons after application of each blocker did not alter significantly (CNQX: 27±9 % vs. 22±9 %, DAPV: 46±4 % vs. 38±6 %, Figure 4.7B right), thus any persistent network activity remained synchronous. The drop in frequency was not due to a subgroup of neurons but mediated by all neurons (example slices: Figure 4.7C, Suppl. Figure 4.5).

Simultaneous blockade of both receptors dramatically reduced the frequency of network activity (0.064±0.005 Hz to 0.004±0.001 Hz, Figure 4.7D, left) and abolished synchronized suprathreshold activity in all slices (46±4 % for aCSF to 0 %, Figure 4.7D, right). Whole-cell patch-clamp recordings were made from stellate cells to assess subthreshold membrane potential changes before and after blockade of all ionotropic glutamate receptors. Clear periodic network bursts of synaptic activity resulting in action potentials were observed in aCSF (blue trace, Figure 4.7E). Kynurenic acid application silenced all rhythmic suprathreshold and subthreshold activity in stellate cells (green trace, Figure 4.7E, n=4, group data not shown), thus, synchronized network events in sMEC and rhythmic activity of stellate cells were silenced by blockade of ionotropic glutamate receptors at this immature stage, similar to glutamatergic regulation in immature neocortical circuits.

Non-synaptic mechanisms modulating network activity

Neocortical synchrony is modulated by gap junctions during the first postnatal week (Blankenship and Feller, 2010; Sun and Luhmann, 2007). Therefore, we tested whether non-synaptic mechanisms affected early sMEC networks. Gap junction blockers selectively reduced frequency of network activity (0.08±0.012 Hz vs. 0.03±0.007 Hz, Suppl. Figure 4.6A) but did not change the number of active (57±6 % vs. 54±6 %) or synchronized neurons (41±7 % vs. 34±6 %, Suppl. Figure 4.6A). Thus, gap junctions contribute to immature sMEC synchronous network activity but given the residual synchrony after pharmacological blockade, are not the driving mechanism.

Prolonged electrical bursting activity in pyramidal neurons depends on extracellular calcium concentrations in hippocampus and entorhinal cortex with decreased levels inducing
Figure 4.7: Ionotropic glutamatergic signaling underlies spontaneous synchronized network activity in immature sMEC. (A) Example traces of individual neurons before (blue, aCSF) and after inhibition of AMPA (dark green) and NMDA receptors (light green). (B) Blockade of AMPA or NMDA receptors decreased overall frequency of activity (paired t-test, CNQX: t(235)=5.93, p<0.0001, DAPV: t(177)=10.98, p<0.0001) but not the proportion of synchronized neurons (paired t-test, CNQX: t(8)=1.07, p=0.31, DAPV: t(6)=1.22, p=0.27). N=9 and 7 slices containing 236 and 178 neurons for CNQX and DAPV experiments respectively. (C) Significant change in frequency occurred across the majority of neurons following application of either ionotropic glutamate receptor blocker. Example experiments shown (CNQX: 2 increasing, 50 decreasing frequency, DAPV: 1 increasing, 40 decreasing frequency). (D) Combined blockade of AMPA and NMDA receptors significantly decreased network activity (left) and abolished all synchronous suprathreshold network activity (right) in sMEC (paired t-test: t(177)=13.01, p<0.0001 and t(6)=11.22, p<0.0001 respectively). (E) Left: Spike profile of developing stellate cell. Right: Blockade of ionotropic glutamate receptors by kynurenic acid (green) also abolished subthreshold excitatory synaptic activity from individual stellate cells.

Therefore, we tested if changing extracellular calcium levels modulated activity across the sMEC network and further, whether it restored downregulated network activity at the end of the second postnatal week. Indeed, we show that while network composition remained stable, increasing extracellular calcium reduces frequency of activity (0.07±0.006 Hz vs. 0.05±0.004 Hz, Suppl. Figure 4.7A, right) while decreasing levels causes an increase of activity levels at P7-12 (0.07±0.006 Hz vs. 0.08±0.006 Hz, Suppl. Figure 4.7A, right) and enhances the low ac-
activity levels at P13/14 – albeit not restoring it completely to peak activity levels (P7-12, 0.003±0.001 Hz vs. 0.010±0.002 Hz, Suppl. Figure 4.7B, right). This effect was not mediated by NMDA receptors (Suppl. Figure 4.7C). Thus, extracellular calcium levels play a role to upregulate suprathreshold network activity but cannot restore synchrony in sMEC at the end of the second postnatal week.

GABAergic modulation of network activity

From the third postnatal week of development onwards strong feedback inhibition is essential for network function and rhythmic activity in sMEC (Couey et al., 2013; Pastoll et al., 2013). Spontaneous network activity in sMEC becomes asynchronous and sparse at the end of the second postnatal week (Figure 4.2). We hypothesized that increased GABA-ergic tone

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Figure 4.8: GABA-A and GABA-B receptor signaling modulates but does not drive network activity. (A) Example traces before and after blockade of GABA-A receptors at P8/9 group and at P14/15 group. (B) Gabazine application increased the amount of active (one-sample t-test, P8/9: t(10)=2.44, p=0.05 and trend at P14/15: t(7)=2.06, p=0.08, top left) and synchronized neurons (one-sample t-test, P14/15: t(7)=2.56, p<0.05 and trend at P8/9: t(10)=1.83, p=0.01, bottom left). No significant change of network event frequency upon Gabazine application (one-sample t-test, P14/15: t(7)=2.56, p<0.05 and trend at P8/9: t(10)=1.83, p=0.01, bottom right). No age-dependent differences of Gabazine’s effect on active or synchronized neurons and network event frequency (independent t-test, t(17)=−0.519, p=0.61, t(17)=−0.62, p=0.55 and t(17)=−0.05, p=0.97 respectively). Frequency of activity was differentially affected by Gabazine depending on age (independent t-test, age effect: t(625)=5.36, p<0.0001, one-sample t-test, condition effect P8/9: t(140)=−2.07, p<0.05 and P14/15: (216)=3.94, p<0.001, top right). (C) GABA-B receptor effects were not developmentally regulated between P7 and P13 (independent t-test: active neurons: t(7)=−1.12, p=0.30, frequency: t(290)=−2.04, p=0.48, synchronized neurons: t(7)=−1.20, p=0.27, network event freq: t(7)=0.10, p=0.93). No significant effect of GABA-B blockade on proportion of active (one-sample t-test, P7: t(3)=−0.21, p=0.85, P13: t(4)=−1.67, p=0.17, top left) or synchronized (one-sample t-test, P7: t(3)=−0.35, p=0.75, P13: t(4)=−1.42, p=0.23, bottom left) neurons. Significant decrease of activity (one-sample t-test, P7: t(143)=−5.35, p<0.0001, p13: t(147)=−2.97, p<0.01, top right) and network frequency (one-sample t-test, P7: t(3)=−3.41, p<0.05 and a trend P13: t(4)=−2.4, p=0.07, bottom right). N-numbers indicated in individual bars indicating slices or neurons and slices in brackets.
and maturation of the GABAergic system could mediate the abolition of synchronous network activity. Thus, to determine the role of GABA, we tested the blockade of both GABA-A and GABA-B receptors during synchronous activity at the start of the second postnatal week and during asynchronous, sparse activity at P13-15 (Figure 4.8A). Blockade of GABA-A receptors increased the number of active neurons within the network at both developmental stages (P8: +9±4 %, P14/15: +12±6 %, Figure 4.8B, top left). Despite an increase in the number of synchronized neurons upon GABA-A blockade (P8/9: +12±7 %, P14/15: +18±7 %, Figure 4.8B, bottom left), the frequency of network events in these synchronized neurons did not increase (P8/9: -0.01±0.01 Hz, P14/15: 0.01±0.02 Hz, Figure 4.8B, bottom right). However, GABA-A receptor blockade did cause a significant increase in overall activity at P14/15 but a decrease at P8/9 (P8/9: -0.002±0.001 Hz, P14/15: +0.014±0.003 Hz, Figure 4.8B, top right) suggesting a developmental increase in an inhibitory effect of GABA-A mediated signaling to modulate network activity.

In juvenile MEC (third postnatal week), metabotropic GABA-B receptors play a role in terminating persistent network activity of layer III pyramidal neurons (Mann et al., 2009). In contrast to GABA-A receptor blockade, GABA-B receptor blockade did not increase the number of active nor synchronous neurons (Figure 4.8C left). However, inhibition of GABA-B receptors decreased the frequency of activity and the rate of synchronous firing at both timepoints measured (P7: 0.03±0.008 Hz, P13: 0.03±0.011 Hz and P7: -0.1±0.003 Hz, P13: -0.007±0.002 Hz, Figure 4.8C right). Thus, GABA-A and GABA-B receptors do not drive synchrony but significantly modulate network activity levels overall by P13/15 and regulate activity in comparable patterns to those reported from the third postnatal week onwards. However, GABA-A and GABA-B effects are mediated in an opposing manner to each other at this immature stage of development.

Discussion

GABAergic inhibition plays a key role in regulating sMEC activity from the third postnatal week of development onwards, when distinct grid cells first appear in vivo (Couey et al., 2013; Langston et al., 2010; Pastoll et al., 2013). In contrast, our data show that prior to spatial exploration and eye-opening in the rodent, intrinsic sMEC network activity is highly synchronous both in vivo and in vitro, and mediated by glutamatergic not GABAergic mechanisms. sMEC synchronous networks occur in a non-random pattern through superficial layers, akin to the functional modules predicted (McNaughton et al., 2006). Synchronized activity is time-locked across both deep and superficial layers in layer V pyramidal neurons and layer II stellate cells and entrains synchronized activity in neighboring neocortex. These synchronized rhythmic patterns of activity are transient, disappearing as GABAergic inhibition matures by the end of the second postnatal week.
Our data clearly show that synchronous sMEC network activity and rhythmic firing of stellate cells is mediated by glutamatergic excitation during the second postnatal week of development. This ‘pre-grid cell’ activity contrasts with the predominant dense recurrent GABAergic inhibition one week later and in young adult rodents (Couey et al., 2013; Pastoll et al., 2013) that has been shown to be sufficient to generate grid cell firing patterns in attractor dynamic models (Couey et al., 2013; Pastoll et al., 2013). Mature entorhinal cortex has a clear modular organization, with dendritic and axonal clusters of 400-500 µm projecting from deeper layers into layer II (Hevner and Wong-Riley, 1992; Ikeda et al., 1989; Solodkin and Van Hoesen, 1996). Models of early MEC development predict topographically-organized modules of both excitatory and inhibitory connectivity that form in an experience-independent manner (McNaughton et al., 2006). However, there is a lack of data determining the relevant biological parameters for computational models of MEC development before recurrent inhibition. Here, our findings reveal a synchronized module of neurons non-randomly distributed throughout sMEC layers up to a maximal distance of 450 µm in a developmental period just prior to eye-opening and explorative behavior. In these immature networks, stained synchronized neurons were just over 150 µm apart and significantly closer to each other than to their active but asynchronous neighbors. This distance is comparable to the distribution ranges observed for connectivity maps of stellate cells and for the patch-like distribution of cytochrome oxidase staining in rodent sMEC (Beed et al., 2010; Burgalossi et al., 2011). Thus, we hypothesize that these highly synchronous immature networks of neurons are an early precursor for later formation of patches of functionally connected neurons.

In the adult, excitatory drive from the hippocampus to sMEC modulates grid cell function (Bonnevie et al., 2013) and long-range hippocampal GABAergic projections alter rhythmic network activity in MEC (Melzer et al., 2012). Connectivity between parahippocampal regions of pre- and parasubiculum to MEC exists by P10 (Canto et al., 2012). Thus, by P10 MEC receives extrinsic inputs. However, at peak network activity and synchrony ages, we observed only weak and unreliable functional long-range connections from hippocampus proper to immature sMEC. In the adult brain, high levels of synchrony occur between MEC and CA1 hippocampus (Hahn et al., 2012) whereas we show that in immature brain, both DG and CA1 were synchronized only approximately 25 % of the time with sMEC network bursts compared to 79 % synchrony between MEC and neocortical regions at the same age. Further, we demonstrate that in contrast to the driving influence of hippocampus onto adult MEC (Bonnevie et al., 2013; Melzer et al., 2012), stimulating hippocampal fibers directly did not induce bursting activity in immature MEC.

The entorhinal cortex is proposed to act as a pacemaker for cortical Early Network Oscilla-
tions (cENOs) during the first postnatal week (Garaschuk et al., 2000; Namiki et al., 2013). Our results show that MEC is indeed pacing the rate of rhythmic activity in neighboring neocortex during the second postnatal week since lesioning between the two regions caused a decrease, but no disappearance of neocortical activity alone. Further, the high level of synchrony between MEC and neocortex greatly reduced after the lesion. The residual NeoC activity could be intrinsic to the local circuit, potentially entrained by earlier prenatal subplate activity (Hanganu et al., 2009).

Due to strong connectivity from deep to superficial MEC (Beed et al., 2010; Dhillon and Jones, 2000; van Haeften et al., 2003) we anticipated that deep layers would drive spontaneous rhythmic activity in superficial networks. However, our data showed strongly time-locked synchrony at both a network level between deep and superficial layers and at individual neuron level between layer V pyramidal and layer II stellate cells. In addition to a lack of delay between network bursts that would indicate directionality, we could not evoke bursting activity by stimulating dMEC or sMEC. We extend the finding in individual layer III neurons that synchronous activity is intrinsic to immature MEC (Sheroziya et al., 2009) to a network level across multiple MEC layers. Both layer V pyramidal and stellate cells receive synchronous synaptic inputs, similar to the common synaptic inputs on neurons across all layers of MEC from pre- and parasubiclar regions at P14-31 (Canto et al., 2012). For synchronous bursts, the origin of this immature activity remains to be determined. In lateral entorhinal cortex during the first postnatal week, superficial neurons drive bursting activity within deeper layers with second-long timelags (Namiki et al., 2013). However, we see no evidence for significant timelags between deep and superficial layers. It remains to be determined whether hub neurons exist in MEC that could coordinate or induce network activity, similar to GABAergic interneurons in developing hippocampus (Bonifazi et al., 2009).

GABAergic inhibition mediates rhythmic synchronized network activity within immature rodent cerebellum (P4-6, Watt et al., 2009) and hippocampal CA3 and CA1 regions, categorized in hippocampus as giant depolarizing potentials (GDPs, P3-12, Ben-Ari et al., 1989; Garaschuk et al., 1998; Khazipov et al., 2004). In contrast to the hippocampus, glutamatergic network mechanisms and synchrony in immature sMEC are similar to those underlying early synchrony throughout neocortex during the first postnatal week (cENOs, Conhaim et al., 2011; Garaschuk et al., 2000; Namiki et al., 2013). The loss of spontaneous network activity upon inhibition of AMPA and NMDA receptors could be explained by a loss of excitatory input to GABAergic interneurons that potentially drive activity within the network. However, blocking GABA-A receptors directly did not decrease the fraction of active nor synchronized neurons within either age group tested but rather increased the numbers of ac-
tive and synchronized neurons. Thus, despite the strong reciprocal connectivity of parahippocampal regions, including MEC itself, to the hippocampus, distinct mechanisms underlie immature synchrony in these circuits.

We find that suprathreshold network synchrony of MEC is tightly developmentally regulated and transient. Peak measures of synchronous activity and maximal levels of synchronized neurons at P10/11 correspond to those observed in somatosensory and visual cortices prior to eye-opening in rodents in vivo (Golshani et al., 2009; Rochefort et al., 2009; Siegel et al., 2012). What curtails suprathreshold network activity within MEC? By the end of the second postnatal week, we observed sparse and largely asynchronous sMEC network activity that is modulated by GABA-A receptor blockade – opposite to that observed one week earlier. Although inhibition of GABA-A receptors and altered extracellular calcium waves can upregulate network activity, in agreement with recordings from individual neurons in MEC and neocortex (Conhaim et al., 2011; Sheroziya et al., 2009), neither was able to restore synchronized activity across the network. This inhibition of network activity by P14/15 concurs with an upregulated inhibitory network reported in sMEC from the third postnatal week onwards in rodents and reflects functional circuitry in the adult (Couey et al., 2013).

What is the function of early synchronous activity in MEC? Synchronous activity is correlated with synapse maturation and sensory map formation (Blankenship and Feller, 2010) and disruption causes aberrant maturation of circuits (Grubb et al., 2003; McLaughlin et al., 2003; Mrsic-Flogel et al., 2005). Thus, we postulate that rhythmic waves of MEC activity play a key role in experience-independent maturation of local circuitry, entraining the network for the abrupt appearance of grid cells during the third postnatal week (Wills et al., 2012). Furthermore, we speculate that the early synchronously active modules we describe are predecessors of the cross-layer, anatomically-overlapping functional clusters of grid cells in adult (Hafting et al., 2005; Stensola et al., 2012).
Supplementary Figure 4.1: Identified stellate cells within synchronous MEC modules. (A) Targeted whole-cell patch-clamp recordings of immature stellate cells filled with Alexa594 (red) within synchronous modular networks. White arrow: same stellate cell as middle, Black arrow: stellate cell indicated. (B) Example step profile of immature stellate cell recorded. Inset: Doublet spike characteristic for stellate cells. Scalebars: 40 µm. (C) Comparison between frequency of rhythmic, synchronized activity of synaptic currents recorded in stellate cells (P8-10, n=6), stellate cell AP spiking activity (P8-10, n=14), LFP in vitro in sMEC (P7-11, n=23) and LFP in vivo in sMEC (P7-8, n=4 pups).
Supplementary Figure 4.2: Spatial distributions of silent, active and synchronized neurons in superficial MEC networks. Individual contour maps of networks at postnatal age 8-10 (n=18 slices) coding synchronized (green), active but not synchronized (blue) and silent (red) neurons. In many networks, a distinct spatial cluster of synchronized neurons is observed e.g. row 2, column 1, whereas other synchronized networks are spatially diffuse e.g. row 1, column 3.
Supplementary Figure 4.3: Spontaneous synchronized network activity is intrinsic to the developing MEC. (A) Horizontal brain slice before and after MEC dissection. (B) Lesion causes no change in the number of active or synchronized neurons in sMEC (paired t-test, t(9)=-0.02, p=0.983 and t(9)=0.23, p=0.82). (C) Isolation of MEC from hippocampus, pre- and parasubiculum and neocortex does not cause a significant decrease in frequency of activity (paired t-test, t(9)=1.95, p=0.08) but in frequency of synchronized events (paired t-test, t(9)=2.34, p<0.05). Number of slices tested indicated on individual bars.

Supplementary Figure 4.4: Field recordings of dMEC-sMEC synchrony and stimulation experiments. (A) Photograph of typical MEA positioning overlaid with field electrode locations in deep and superficial layers. (B) Cell-attached field recordings in deep and superficial MEC layers. (C) Histogram of the observed time-lag for event onset using high temporal resolution field recordings shows correlated synchrony between layers but no directionality of activity (one-sample t-test, t(7)=0.50, p=0.63) in immature MEC. N=7 slices. (D) Stimulation in dMEC does not induce network bursts in sMEC layers (chi square with Yates correction, chi square(1)=0.1, p=0.67). Stimulation in dMEC does not induce local network bursts in dMEC (chi square with Yates correction, chi square(1)=3.21, p=0.07). N=600 stimulations.
Supplementary Figure 4.5: No evidence of a subgroup of neurons responsible for glutamate effect. (A) Upon both AMPA-R and NMDA-R blockade the vast majority of neurons remained silent or active and only minor proportions changed activity states i.e. became active or lost activity indicating that the effect of glutamatergic blockers is not mediated by a subgroup of neurons pacing the network.

Supplementary Figure 4.6: Synchronized network activity modulated by gap junctions. (A) Blockade of gap junctions does not change the proportion of active and synchronized neurons (paired t-test, t(12)=0.84, p=0.41, and t(12)=1.74, p=0.11, respectively, left). Frequency of activity and network events is significantly lowered upon CNX application (paired t-test, t(919)=23.65, p<0.0001, top right, t(12)=5.25, p<0.001, bottom middle). (B) Application of the CNX analog GZA that possesses the same side-effects but does not block gap junctions did not result in any significant decreases (paired t-test, active: t(4)=0.44, p=0.69, synchronized: t(4)=0.84, p=0.45, network event freq: t(4)=0.17, p=0.87, but in a small increase in frequency (paired t-test, t(378)=2.35, p<0.05, top right). \( N \) indicated in individual bars.
Supplementary Figure 4.7: Age-dependent modulation by extracellular calcium concentration.

(A) Example traces of an individual neuron in different extracellular calcium concentrations in group P712 (left). Changing calcium levels did not alter the amplitude of individual network events thereby altering activity detection thresholds (data not shown). No change in proportion of synchronized neurons (middle) in different extracellular calcium concentrations (one-way ANOVA, F(2,18)=0.23, p=0.794) but a significant negative regulation of frequency (right) in higher extracellular calcium concentrations (Bonferroni corrected, 0.6 mM vs. 2.6 mM: 0.08±0.006 Hz vs. 0.5±0.004 Hz, p<0.01, one-way ANOVA, F(2,285)=6.72, P<0.01, trend (Bonferroni corrected): 1.6mM vs. 2.6mM: 0.07±0.006 Hz vs. 0.05±0.004 Hz, p=0.06). (B) Example trace of an individual P13/14 group neuron (left). No changes in proportion of synchronized neurons upon Ca^2+ changes (paired t-test, t(11)=1.13, p=0.28, middle) but a threefold increase in frequency upon decrease of extracellular Ca^2+ from 1.6 mM to 0.6 mM (paired t-test, t(187)=3.38, p<0.001, right). (C) Significant increase in activity levels in decreased extracellular Ca^2+ level persists in the presence of NMDA receptor blocker DAPV (frequency (1.6 mM Ca^2+ + DAPV vs. 0.6 mM Ca^2+ + DAPV): 0.011±0.002 Hz vs. 0.036±0.004 Hz, Bonferroni corrected: p<0.0001, one-way ANOVA, F(2,666)=22.10, p<0.0001; network event frequency (1.6mM Ca^2+ + DAPV vs. 0.6mM Ca^2+ + DAPV): 0.006±0.003 Hz vs. 0.061±0.014 Hz, Bonferroni corrected: p<0.01, one-way ANOVA, F(2,18)=8.01, p<0.01) while leaving the number of active and synchronized neurons undisturbed (active neurons: one-way ANOVA, F(2,18)=1.99, p=0.166; synchronized neurons: one-way ANOVA, F(2,18)=0.85, p=0.442). N-numbers indicated in individual bars (number of slices for synchrony and number of neurons and slices in brackets for frequency).
References


