Spontaneous Bursting Activity in the Developing Entorhinal Cortex

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Periodic spontaneous activity represents an important attribute of the developing nervous system. The entorhinal cortex (EC) is a crucial component of the medial temporal lobe memory system. Yet, little is known about spontaneous activity in the immature EC. Here, we investigated spontaneous field potential (fp) activity and intrinsic firing patterns of medial EC layer III principal neurons in brain slices obtained from rats at the first two postnatal weeks. A fraction of immature layer III neurons spontaneously generated prolonged (2–20 s) voltage-dependent intrinsic bursting activity. Prolonged bursts were dependent on the extracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_o$). Thus, reduction of $[\text{Ca}^{2+}]_o$ increased the fraction of neurons with prolonged bursting by inducing intrinsic bursts in regularly firing neurons. In 1 mM $[\text{Ca}^{2+}]_o$, the percentages of neurons showing prolonged bursts were 53%, 81%, and 29% at postnatal day 5 (P5)–P7, P8–P10, and P11–P13, respectively. Prolonged intrinsic bursting activity was blocked by buffering intracellular Ca^{2+} with BAPTA, and by Cd^{2+} , flufenamic acid (FFA), or TTX, and was suppressed by nifedipine and riluzole, suggesting that the Ca^{2+} -sensitive nonspecific cationic current (I_{CAN}) and the persistent Na^+ current (I_{Nap}) underlie this effect. Indeed, a 0.2–1 s suprathreshold current step stimulus elicited a terminated plateau potential in these neurons. fp recordings at P5–P7 showed periodic spontaneous glutamate receptor-mediated events (sharp fp events or prolonged fp bursts) which were blocked by FFA. Slow-wave network oscillations become a dominant pattern at P11–P13. We conclude that prolonged intrinsic bursting activity is a characteristic feature of developing medial EC layer III neurons that might be involved in neuronal and network maturation.

Introduction

Periodic spontaneous activity is a characteristic feature of the developing nervous system. It is believed that early spontaneous activity is involved in the modulation of several processes during brain maturation, including neuronal growth, synapse formation, and network construction (Ben-Ari, 2002). Initially described in the hippocampus using intracellular recordings and termed giant depolarizing potentials (GDPs) (Ben-Ari et al., 1989), the periodic spontaneous network events have also been observed in a wide range of developing brain structures, including cortex, spinal cord, and retina (O'Donovan, 1999; Feller, 1999; Garaschuk et al., 2000). Despite basic similarity of the phenomena, the patterns of synchronous activity as well as the mechanisms underlying spontaneous events might be highly diverse depending on the brain structure and developmental stage (Feller et al., 1996; Leinekugel et al., 1997; Kandler and Katz, 1998; Dupont et al., 2006; Crépel et al., 2007; Allène et al., 2008). In the

immature cortex, spontaneous electrical activity and large-scale oscillatory calcium waves have been observed both *in vitro* and *in vivo* (Yuste et al., 1992; Garaschuk et al., 2000; Corlew et al., 2004; Khazipov et al., 2004; Adelsberger et al., 2005; Hanganu et al., 2006; Khazipov and Luhmann, 2006). Recently, four distinct patterns of early spontaneous activity, differing by their developmental profile, calcium dynamics, and mechanisms, were defined in the immature cortex: uncorrelated calcium spikes, cortical synchronous plateau assemblies (cSPAs) nesting recurrent bursts via gap junction coupling, glutamate-driven cortical early network oscillations (cENOs) (Garaschuk et al., 2000), and a cortical pattern corresponding to GABA-driven GDPs (cGDPs) (Allène et al., 2008).

It is generally accepted that the early excitatory actions of GABA have a central role in the generation of GDPs (Ben-Ari et al., 2007), whereas cENOs are generated by activation of glutamatergic synapses mostly through NMDA receptors (NMDARs) (Allène et al., 2008). However, the hypothesis that the mechanism of rhythmogenesis for early network oscillations could involve the activity of cell autonomous pacemakers in a recurrent synaptic network is under investigation. Recently, it has been shown that intrinsic bursting of immature starburst cells underlies the generation of retinal waves (Zheng et al., 2006). Moreover, neurons displaying intrinsic rhythmic activity were described in the developing hippocampus (Sipilä et al., 2005; Crépel et al., 2007).

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However, the existence of pacemakers driving early network patterns in developing central structures is still unclear.

The entorhinal cortex (EC) is a crucial component of the medial temporal lobe memory system (Squire et al., 2004). The EC constitutes the major interface between the hippocampus and the parahippocampal cortex and is subdivided into a medial area (mEC) and a lateral area (lEC). The EC exhibits complex extrinsic and intrinsic connections (for review, see van Strien et al., 2009). Thus, early spontaneous activity in the EC might be involved in functional and structural development of hippocampal and cortical networks. Yet, little is known about early spontaneous activity within the EC. Here, we investigated spontaneous activity in layer III (LIII) of the immature mEC by using whole-cell, cell-attached, and field potential (fp) recordings in horizontal brain slices.

Materials and Methods

Preparation of brain slices. Horizontal brain slices (500–600 μm thick) containing the hippocampus and entorhinal and parts of perirhinal cortices were obtained from Wistar rats at the first two postnatal weeks [postnatal day 1 (P1)–P13] by using standard procedures. P0 was taken as the day of birth. All experimental protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the state government of Baden-Württemberg. Animals were decapitated, and brains were rapidly removed and placed in a cold (4–6°C) oxygenated artificial CSF (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.6 CaCl_2 , 1.8 MgSO_4 , 26 NaHCO_3 , 1.25 NaH_2PO_4 , and 10 glucose (for interface recording) or containing 130 NaCl, 3.5 KCl, 1.2 NaH_2PO_4 , 25 NaHCO_3 , 1.3 MgCl_2 , 1 or 2 CaCl_2 , and 25 glucose (for submersion-type recording). Then, brains were saturated with 95% O_2 and 5% CO_2 , pH 7.4. Brain slices were cut using a manual Vibratome slicer (Campden Instruments) and were then incubated in a holding chamber for at least 1 h at room temperature. Individual slices were transferred into a recording chamber one by one, superfused with ACSF at a rate of 1–3 ml/min, and maintained at $33 \pm 1^\circ\text{C}$. For extracellular fp recordings, slices were allowed to recover in a recording chamber for at least 1 h before starting a recording session.

Recording procedures. Whole-cell patch-clamp recordings were made under visual guidance using an Olympus microscope fitted with infrared differential interference contrast optics (Olympus BX51WI). The lamina dissecans (sometimes referred to as layer IV) was used as a reference of the deep border of LIII. Pyramidal neurons located in deeper part of LIII were preferentially recorded. Current-clamp recordings were performed with an Axopatch 1D patch-clamp amplifier (Axon Instruments) in a submersion-type recording chamber. Patch electrodes were backfilled with the following (in mM): 115 K-gluconate, 20 KCl, 10 disodium phosphocreatine, 10 HEPES, 4 MgATP, and 0.3 GTP (tip resistance of 10–12

Figure 1. Periodic spontaneous field activity in the developing mEC. Characteristic spontaneous fp activity in the mEC LIII recorded in slices at P1–P4 (**A**), P5–P7 (**B**), P8–P10 (**C**), and P11–P13 (**D**) (low-pass filter at 25 Hz). Right, Events marked on the left are shown at an expanded time scale (raw data). **A**, Individual fp events accompanied with unit discharges detected in a P2 slice. **B**, Periodic spontaneous fp activity recorded at P5–P7 is characterized by a selective presence of sharp fp events (**a**, trace at P5), prolonged fp events accompanied by fp fluctuations (“fp bursts”) (**b**, trace at P7), or of both forms simultaneously (**c**, trace at P6). **Bc**, Right, Schematic representation of the horizontal hippocampal–EC slice with the position of recording electrode. **C**, Periodic fp events recorded at P8–P10. Note the increase of network rhythmicity for sharp fp events (**a**, P10) and fp bursts (**b**, P8). **D**, Typical spontaneous slow-wave network activity at P11–P13 (specimen trace at P11). **E**, Bar diagrams summarizing the mean durations and peaks of amplitude of fp events at four sequential postnatal periods. Error bars indicate SD. * $p < 0.05$, *** $p < 0.005$.

M Ω). The electrode solutions were adjusted to pH 7.2 with 1 M KOH. Data were low-pass filtered at 1 kHz, digitized at 5–10 kHz, and stored on a personal computer using the LTP230 software package (courtesy of W. W. Anderson, Bristol University, Bristol, UK) (Anderson and Collingridge, 2001). Extracellular fp recordings were performed with an Axoclamp-2B amplifier (Axon Instruments) and a DPA-2FX extracellular amplifier (NPI Electronics) in an interface recording chamber (Fine Science Tools). Signals were filtered on-line at 1 kHz and digitized at 5–10 kHz by an analog to digital converter 1401 MICRO [Cambridge Electronic Design (CED)]. Extracellular signals were stored on a computer using Spike2 software (CED). fp recordings were obtained with

ACSF-filled glass capillary electrodes (tip resistance of $\sim 1\text{ M}\Omega$) placed in LIII of the mEC. The EC was identified with a dissecting microscope by transillumination. An extracellular bipolar electrode (SNEX-200, Rhodes Medical Instruments) was used to induce synaptic fp responses by local stimulation of the IEC. Pulses (0.1 ms, 5–25 V) were delivered with an Iso-Flex stimulus isolator (AMPI) triggered by a Master-8 VP pulse generator. Parts of stimulation artifacts were eliminated in the represented traces. Experiments on the “isolated EC” (isol-EC) were performed on slices containing both lateral and medial parts of the EC.

Chemicals. Flufenamic acid (FFA, 10, 20, 50 μM), riluzole (5, 10 μM), nifedipine (10, 50, 100 μM), mefloquine (25, 50 μM), tetraethylammonium chloride (TEA, 1 mM), tetrodotoxin (TTX, 1 μM), atropine (10 μM) (all from Sigma-Aldrich), and CdCl_2 (100 μM), NiCl_2 (100 μM), and E4CPG (200 μM , Tocris Cookson) were bath applied by continuous perfusion. FFA and riluzole were applied from stock solutions made in DMSO. The final concentration of DMSO in ACSF was $\leq 0.1\%$. Control experiments revealed no measurable effects of DMSO on cellular properties or network periodic events ($n = 5$). 1,2-Bis(2-aminophenoxy)ethane- N,N,N',N' -tetra-acetic acid (BAPTA, 30 mM, Sigma-Aldrich) was applied intracellularly through the patch pipette solution for 10–20 min. BAPTA solution (30 mM) contained the following (in mM): 30 $\text{K}_4\text{-BAPTA}$, 50 sucrose, 20 KCl, 10 disodium phosphocreatine, 10 HEPES, 4 MgATP, and 0.3 GTP. The solutions were adjusted to pH 7.2 with 1 M KOH. Blockade of ionotropic glutamate receptor (iGluR) and GABA $_A$ receptor (GABA $_A$ R)-mediated neurotransmission was performed with a drug mixture consisting of either a mixture of kynurenic acid (2 mM) and picrotoxin (100 μM) or a mixture of DL-2-amino-5-phosphonovaleric acid (APV, 60 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 30 μM), and picrotoxin (100 μM , all from Sigma-Aldrich, Taufkirchen, Germany). To investigate the role of GABAergic transmission in generation of fp activity, we applied picrotoxin (20 μM) in the bath for 10 min, and then the drug was washed out for ~ 60 min. In our experiments, the slices were superfused with ACSF containing 2, 1.6, or 1 mM Ca^{2+} ($[\text{Ca}^{2+}]_o$), which in bicarbonate-based solution corresponds in fact to lower values. Thus, by using 1.6 mM $[\text{Ca}^{2+}]_o$, the real free extracellular Ca^{2+} concentration in bicarbonate-based ACSF is ~ 1.2 mM (Yue et al., 2005).

Data analysis. Electrophysiological data were analyzed off-line using Spike2 software (CED). fp activity at P1–P4 was analyzed from 10 min recordings in each of 40 slices (10 slices from four rats for each postnatal day). fp activity at P5–P7 was analyzed from at least 5 min of primary data. Duration of fp events (DE) was measured from the onset of negativity until the peak of positivity before the field waveform return to the baseline level. The peak amplitude of fp events (PA) was calculated as the difference from baseline to peak of the field waveform. The preferred frequency of events (pFE) was calculated as a peak in the distribution of interevent intervals. In illustrations, field potential recordings were low-pass filtered at 25 Hz. Effect of lowering $[\text{Ca}^{2+}]_o$ on fp events was analyzed from 5 min of recordings (control vs 20–30 min perfusion of ACSF containing 1 mM Ca^{2+}). fp activity at P8–P13 was calculated from 1 min recordings. Spontaneous paroxysmal field discharges elicited by picrotoxin were calculated from 1 min recordings at a period with the maximal amplitude of discharges. “Amplitude” of discharges and seizure-like events was defined as the difference between the most negative and most positive data points during an event, and duration was measured from the onset of negativity until the peak of positivity before the fp return to the baseline. Spontaneous intrinsic firing activity was analyzed from at least 2 min of recordings. Bursting activity in the presence of 100 μM nifedipine was recorded and analyzed after depolarization of membrane potential to -52 ± 3 mV versus -60 ± 1.2 mV in control recordings. Burst duration was calculated as the time between the first spike of a burst and the last spike of that burst. Interburst interval was defined as the point from the end of a burst to the beginning of the next burst. Plateau duration was calculated as the time between the end of a current step and the last spike of the plateau. Because no significant differences in duration, amplitude and frequency of events were detected for combined EC-hippocampal (EC-H) versus isol-EC slices at P8–P10 and P11–P13, data were pooled from both groups of slices. For calculating the effects of APV or CNQX at P5–P7, data were summarized from sharp fp events

Table 1. Quantitative parameters of the fp events at different postnatal periods

Period	Peak of amplitude (mV)	Duration (s)	Frequency (Hz)
P1–P4			
fp events in mEC ($n = 6$)	0.03 ± 0.01	0.7 ± 0.3	~ 0.004
fp events in CA3 ($n = 29$)	0.09 ± 0.06	0.7 ± 0.2	0.07 (pFE)
P5–P7			
Selective presence			
fp-sharp ($n = 34$)	0.16 ± 0.1	0.9 ± 0.36	0.08 (pFE)
fp bursts ($n = 11$)	0.1 ± 0.08	3.5 ± 1.2	0.06 (pFE)
Simultaneous presence ($n = 8$)			
For fp-sharp	0.08 ± 0.05	1.19 ± 0.5	
For fp bursts	0.09 ± 0.06	3.5 ± 2	
P8–P10			
fp-sharp ($n = 4$)	0.08 ± 0.01	1.7 ± 0.3	0.16 ± 0.1
fp bursts ($n = 22$)	0.03 ± 0.02	3.2 ± 0.8	0.1 ± 0.05
P11–P13			
fp bursts ($n = 37$)	0.04 ± 0.02	4.8 ± 2.5	0.14 ± 0.06

Averaged data are given as mean $\pm$ SD. Recordings were made in ACSF containing 1.6 mM Ca^{2+} . At P1–P4, 1–4 events per 10 min were detected in the mEC LIII. At P5–P13, all recordings were made in the mEC LIII. For P5–P7, parameters are calculated for slices with selective presence of sharp fp events or fp bursts and for slices with simultaneous appearance of both forms of fp activity.

(fp-sharp) and fp bursts. Frequency of the occurrence of fp events at P5–P7 was calculated from 5 min of recordings under control conditions and in the presence of APV or CNQX. NMDA and AMPA/kainate receptor-mediated components of synaptic responses as well as antidromic response were identified on the basis of their selective blockade with APV, CNQX, and TTX (see supplemental Fig. 5, available at www.jneurosci.org as supplemental material). For each component the amplitude was calculated as the difference from baseline to peak of the field waveform. Since influence of FFA on intrinsic neuronal excitability may also indirectly affect synaptic responses, we used for the analysis only experiments in which antidromic responses were not significantly changed by FFA (five of seven tested slices). In the remaining two slices FFA reduced amplitude of antidromic components to $73 \pm 2\%$, AMPA/kainate components to $72 \pm 0.4\%$, and NMDA components to $46 \pm 3\%$ of the control values. Averaged data are given as mean $\pm$ SD. Paired or unpaired two-tailed Student's t test or Wilcoxon–Mann–Whitney rank sum test was used for statistical comparison.

Results

We investigated spontaneous activity in the immature mEC by using field potential recordings in LIII and whole-cell patch-clamp recordings from LIII principal neurons in horizontal brain slices.

Periodic spontaneous field activity in the developing mEC

Extracellular fp recordings were performed in slices obtained from rats at P1–P13. We first determined the patterns of spontaneous fp activity at four sequential postnatal periods: (P1–P4), (P5–P7), (P8–P10), and (P11–P13). All fp recordings were made in 1.6 mM $[\text{Ca}^{2+}]_o$.

We found that at P1–P4, spontaneous fp activity in the mEC was poorly expressed. In 25 of 40 recorded slices (63%) periodic spontaneous fp activity in the mEC was not identified. In 6 of 40 slices (15%, slices at P2–P4) only individual fp events (1–4 events per 10 min) were detected. These fp events were characterized by negative fp deflection and were accompanied by unit discharges (Fig. 1A). In the remaining nine slices (22%) only individual unit bursts without fp shifts were observed. In contrast, simultaneous control fp recordings from CA3 area of the hippocampus at P1–P4 showed periodic spontaneous fp events in the majority of tested slices (32 of 38; 84%), similar to what has been reported by Sipilä and colleagues (2005) (Table 1).

The fp recordings in the mEC at P5–P7 demonstrated periodic spontaneous field events that were characterized by ~ 0.5 –5 s

negative-going shifts in the range of ~ 0.02 – 0.46 mV (sharp fp events or prolonged fp events accompanied by field fluctuations) (Fig. 1*B*). fp events were associated with multiple unit discharges. In the majority of the recorded slices (34 of 53; 64%) spontaneous fp activity displayed selective sharp fp events as illustrated in Figure 1*Ba*. Prolonged fp events accompanied by fp fluctuations at ~ 15 – 30 Hz (termed “fp bursts”) were observed in 21% of the recordings (Fig. 1*Bb*). In the remaining eight slices (15%) both forms of fp activity were detected (Fig. 1*Bc*). Periodic fp activity in the mEC was still present after surgical separation of the hippocampus ($n = 25$) (supplemental Fig. 1*A*, supplemental text, available at www.jneurosci.org as supplemental material), suggesting an involvement of local neuronal circuits within the EC in this phenomenon. Electrical stimulation of the lateral EC (IEC, 0.1 ms, 6–17 V) was sufficient to induce sharp fp events or fp bursts, similar to those occurring spontaneously (Fig. 2*Aa*). fp events could be equally evoked in combined EC-H slices ($n = 8$) as well as in isol-EC slices ($n = 4$).

fp recordings during the second postnatal week revealed a noticeable alteration in the proportion of sharp fp events versus fp bursts. Thus, at postnatal days 8–10, only a minority of slices (4 of 26; 15%) displayed principal activity that could be distinguished as sharp fp events (Fig. 1*Ca*). In 22 of 26 slices (85%) the fp recordings showed rhythmic occurrence of fp bursts (6 ± 3 fp bursts/min) (Fig. 1*Cb*). Data were summarized from 18 EC-H and 8 isol-EC slices. We also observed superimposition (i.e., simultaneous appearance) of sharp fp events and fp bursts (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material).

Spontaneous fp activity at P11–P13 displayed characteristic slow-wave network rhythmicity (Fig. 1*D*) in the majority of slices (37 of 43; 86%; 23 EC-H and 20 isol-EC slices). The pattern was characterized by regular repetitive fp bursts. When stabilized, the frequency of the occurrence of fp bursts was 8.6 ± 3.6 events/min. In 6 of 43 slices we observed just continuous fp fluctuations at ~ 20 – 40 Hz, and we were not able to separate individual events. In addition, individual negative fp deflections [range: 0.5 ± 0.12 s, 0.11 ± 0.1 mV; 1–5 deflections/10 min ($n = 34$)] were simultaneously detected in 13 of 22 and 24 of 37 slices displaying fp bursts at P8–P10 and P11–P13, respectively. Similar to P11–P13, fp activity was also observed in juvenile rats (tested at P14–P18; $n = 8$). Interestingly, comparable slow-wave oscillations (SWOs) were reported in the EC of adult rats (Cunningham et al., 2006). The quantitative parameters of fp events at different postnatal periods are summarized in Table 1.

Figure 2. Role of glutamatergic neurotransmission in generation of periodic spontaneous field activity in the mEC in slices obtained from rats at P5–P7 (**A**) and P11–P13 (**B**). **Aa**, Electrical stimulation of the lateral EC (indicated by arrowheads; left trace, 17 V; right trace, 12 V) is sufficient to induce sharp fp events or fp bursts that are comparable to the spontaneous fp events. Right, Durations and peak amplitudes of spontaneous and evoked sharp fp events ($n = 5$) and fp bursts ($n = 7$). **Ab, c**, Spontaneous (top) and stimulus-evoked (bottom) fp events (stimuli, 8 and 15 V for **b** and **c**, respectively; indicated by arrowheads) in control and during partial and full blockade of iGluRs. The occurrence of spontaneous fp events is strongly reduced in the presence of APV (60 μ M) or CNQX (30 μ M) and is completely blocked after adding of the mixed NMDA/AMPA/kainate receptor antagonists. Note that strong, but not weak, electrical stimulations are still able to induce fp events in the presence of APV or CNQX (the specimen traces of spontaneous and evoked fp events in the presence of CNQX are shown from different slices). Spontaneous fp activity increases during enhanced tonic excitation following elevation of $[K^+]_o$ and is completely blocked after the addition of CNQX and APV. **B**, Spontaneous slow-wave rhythmic fp activity recorded at P11–P13 is modified after separate adding of APV (**a**) or CNQX (**b**) and is fully blocked in the presence of both drugs. **Bc**, Duration, peak amplitude, and frequency of fp bursts during slow-wave activity under control conditions and after adding APV or CNQX. Averaged data are given as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. ns, Not significant.

We further investigated the role of ionotropic glutamatergic and GABAergic neurotransmissions in the generation of periodic spontaneous fp events in the developing mEC. At P5–P7, bath application of the NMDAR antagonist APV (60 μ M) strongly suppressed the periodic spontaneous fp events (both fp-sharp and fp bursts) (seven of nine slices) (percentage of control values; DE: $55 \pm 24\%$, $p < 0.02$; PA: $109 \pm 41\%$, $p = 0.6$; frequency of the occurrence: $13 \pm 7\%$, $p < 0.005$). Simultaneously, APV prevented appearance of fp events evoked by weak electrical stimulations (6–10 V). However, in four of six slices an increase in the stimulus intensity (to ~ 15 V) was able to induce fp events in the

Figure 3. *A*, Intrinsic firing patterns of developing mEC LIII principal neurons. *Aa*, Prolonged intrinsic bursting activity of LIII neurons recorded at different membrane potentials. Right, Corresponding histograms of the membrane potential distribution calculated for 160 s show two distinct peaks (bin size, 1 mV). Regular firing pattern (*Ab*) and “short-bursting” pattern (*Ac*) of LIII neurons. *Ac*, Right, Membrane potential histograms for the short-bursting pattern were calculated for 60 s of recording (bin size, 1 mV). All recordings were made in 2 mM $[Ca^{2+}]_o$. *B*, Effects of lowering $[Ca^{2+}]_o$ on the intrinsic firing pattern of developing mEC LIII neurons. *Ba*, Switch of regular firing pattern to prolonged-bursting mode after reduction of $[Ca^{2+}]_o$ from 2 to 1 mM. *Bb*, Strengthening of bursts after lowering $[Ca^{2+}]_o$ by neurons displaying prolonged bursting activity in both $[Ca^{2+}]_o$ concentrations. All recordings were obtained in the presence of kynurenic acid and picrotoxin. *C*, Percentage of cells showing prolonged bursting, short bursting, and regular firing activity plotted for the three age groups (P5–P7, P8–P10, and P11–P13). $[Ca^{2+}]_o$ concentrations indicated on the top of the histogram.

presence of the drug (DE: $70.5 \pm 15\%$ of control, PA: $97 \pm 19\%$ of control) (Fig. 2*Ab*). Similar results were obtained by selective blockade of AMPA/kainate receptors with CNQX (30 μ M) (Fig. 2*Ac*). CNQX reduced periodic spontaneous activity in five of six cases (percentage of control values; DE: $76 \pm 21\%$, $p = 0.07$; PA: $112 \pm 33\%$, $p = 0.46$; frequency of the occurrence: $11 \pm 6\%$, $p < 0.005$). CNQX also blocked fp events evoked by weak but not by strong stimulations ($n = 3$ of 4; DE: $73 \pm 13\%$ of control; PA: $64 \pm 47\%$ of control). Subsequent bath application of mixed NMDA/AMPA/kainate receptor antagonists completely blocked spontaneous fp activity in all tested slices ($n = 10$) and always abolished fp events induced by strong stimulations ($n = 8$) (Fig. 2*Ab,Ac*). Further increase in the stimulus intensity (tested up to 40 V) was unable to reinduce fp events in the presence of APV and

CNQX ($n = 8$). In a minority of cases spontaneous fp events were fully blocked by separate applications APV (two of nine) or CNQX (one of six). All effects of APV and CNQX were reversible after washout of the drugs (proved in six slices). In all tested slices at P5–P7 ($n = 5$), a selective blockage of GABA_A receptors with picrotoxin (20 μ M) reversibly elicited spontaneous paroxysmal field discharges (supplemental Fig. 2*A*, available at www.jneurosci.org as supplemental material), as previously described in the neocortex (Wells et al., 2000), indicating an inhibitory contribution for GABAergic neurotransmission in the immature (P5–P7) mEC.

The above data suggested that the iGluR-mediated neurotransmission as well as the general level of glutamatergic excitation is essential for the initiation of periodic spontaneous fp events in the immature mEC. Therefore, we also tested the effect of iGluR antagonists on fp activity in high-extracellular-potassium ($[K^+]_o$) conditions. Influence of elevated $[K^+]_o$ on early network activity was previously investigated in the hippocampus (Sipilä et al., 2005) and the somatosensory cortex (Allène et al., 2008). High $[K^+]_o$ increases the recruitment of NMDARs and reduces the excitatory drive provided by immature GABAergic transmission (Rheims et al., 2008) that can modify network patterns. Thus, we first analyzed the influence of tonic depolarization caused by increasing $[K^+]_o$ on spontaneous fp activity in the immature mEC.

Increase of $[K^+]_o$ to 8 mM for ~20 min resulted in enhanced spontaneous fp activity (Fig. 2*Ad*; for details, see supplemental material at www.jneurosci.org). In all tested slices ($n = 13$) spontaneous fp activity was completely blocked after adding CNQX and APV in high- $[K^+]_o$ conditions (Fig. 2*Ad*). In contrast, bath application of the gap junction blocker mefloquine (25–50 μ M; Cruikshank et al., 2004) failed to block spontaneous fp events

in control ACSF ($n = 6$) and in high potassium ($n = 4$). Thus, in the presence of 50 μ M mefloquine neither sharp fp events (percentage of control values in 8 mM $[K^+]_o$; DE: $99 \pm 42\%$, $p = 0.9$; PA: $87 \pm 9\%$, $p = 0.9$) nor fp bursts (DE: $123 \pm 17\%$, $p = 0.12$; PA: $83 \pm 11\%$, $p = 0.12$) were significantly altered.

We further examined the role of iGluRs and GABA_ARs in the generation of fp activity during the second postnatal week. In all tested P8–P10 slices, spontaneous and evoked fp events ($n = 11$ and 6 respectively) were fully blocked by a mixture of iGluR antagonists, and paroxysmal discharges were observed in the presence of picrotoxin ($n = 8$ EC-H slices). All effects were reversible after washout of the drugs (tested in eight, four, and eight slices for spontaneous, evoked, and paroxysmal fp events, respectively).

Table 2. Quantitative parameters of the prolonged intrinsic bursting activity at different postnatal periods

Parameter (mEC LIII)	2 mM $[Ca^{2+}]_o$		1 mM $[Ca^{2+}]_o$		P8–P10		P11–P13	
	P5–P7	Versus	P5–P7	Versus	P8–P10	Versus	P11–P13	Versus
Number of bursts per minute	2.4 ± 1.8 (<i>n</i> = 7)	ns	2.9 ± 1.2 (<i>n</i> = 14)	***	6.4 ± 3.5 (<i>n</i> = 19)	ns	8.1 ± 2.3 (<i>n</i> = 10)	ns
Burst duration (s)	11.9 ± 7.4 (<i>n</i> = 7)	ns	9.9 ± 6.5 (<i>n</i> = 14)	***	2.9 ± 1.4 (<i>n</i> = 19)	ns	2.5 ± 1.2 (<i>n</i> = 10)	ns
Interburst interval (s)	24.3 ± 15 (<i>n</i> = 7)	ns	17.7 ± 10.5 (<i>n</i> = 14)	*	9.8 ± 6.6 (<i>n</i> = 19)	ns	6.1 ± 3.6 (<i>n</i> = 10)	ns
Burst amplitude (mV)	5.7 ± 2.2 (<i>n</i> = 7)	***	10.3 ± 2.9 (<i>n</i> = 14)	ns	12 ± 2.4 (<i>n</i> = 19)	ns	10.7 ± 4.2 (<i>n</i> = 10)	ns
Spike frequency within bursts (Hz)	2.1 ± 0.5 (<i>n</i> = 7)	***	4.7 ± 1.7 (<i>n</i> = 14)	***	10.9 ± 6 (<i>n</i> = 19)	ns	9 ± 4.9 (<i>n</i> = 10)	ns
Number of spikes per burst	23 ± 12 (<i>n</i> = 7)	*	47 ± 46 (<i>n</i> = 14)	ns	32 ± 26 (<i>n</i> = 19)	ns	22 ± 16 (<i>n</i> = 10)	ns
Spike threshold (mV)	−44.4 ± 2.7 (<i>n</i> = 7)	ns	−41.3 ± 3.4 (<i>n</i> = 10)	**	−47 ± 4.3 (<i>n</i> = 14)	**	−53 ± 4 (<i>n</i> = 8)	**
AHP amplitude (mV)	2.6 ± 0.5 (<i>n</i> = 3)	ns	3.6 ± 1.2 (<i>n</i> = 8)	ns	3.8 ± 1.4 (<i>n</i> = 18)	ns	3.8 ± 1.5 (<i>n</i> = 7)	ns
AHP duration (s)	17.5 ± 3.5 (<i>n</i> = 3)	ns	18.8 ± 17 (<i>n</i> = 8)	***	4.6 ± 3.6 (<i>n</i> = 18)	ns	3.4 ± 2 (<i>n</i> = 7)	ns
Input resistance (GOhm)	1.2 ± 0.1 (<i>n</i> = 4)	ns	1.2 ± 0.3 (<i>n</i> = 11)	***	0.6 ± 0.1 (<i>n</i> = 8)	***	0.4 ± 0.06 (<i>n</i> = 6)	***
Recording membrane potential (mV)	−60 ± 2.5 (<i>n</i> = 7)	ns	−60.5 ± 1.3 (<i>n</i> = 14)	ns	−62 ± 2.6 (<i>n</i> = 19)	ns	−63 ± 2.5 (<i>n</i> = 10)	ns

Averaged data are given as mean ± SD. Recordings were made in ACSF containing 2 mM or 1 mM Ca^{2+} as indicated on the top. iGluRs and GABA_ARs were blocked as detailed in Materials and Methods. Unpaired two-tailed *t* test or Mann–Whitney rank sum test (versus, i.e., left versus right) values are indicated as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.005. ns, Not significant.

In the adult EC, it has been reported that generation of SWOs is mediated by activation of kainate (GluR5) receptors in a recurrent network of pyramidal neurons (Cunningham et al., 2006). Application of CNQX or specific GluR5 receptor antagonist UBP-302 abolished this rhythmic activity in the adult EC. In contrast, we found that CNQX failed to block spontaneous slow-wave network rhythmicity in the mEC at P11–P13 (*n* = 8 of 8). fp activity was significantly affected by APV (*n* = 4) as well as by CNQX (*n* = 5) and was always fully blocked in the presence of both drugs (*n* = 14) (Fig. 2*B*), indicating a conjoined contribution of NMDA and AMPA/kainate receptors in shaping the dynamics of slow-wave network rhythmicity at P11–P13. Blockade of slow-wave activity by a mixture of iGluR antagonists was reversible after washout of the drugs (tested in seven slices). In three of eight experiments with APV, the NMDAR antagonist was able to prevent slow-wave activity independently. Finally, in all tested slices at P11–P13 (*n* = 10; eight isol-EC and two EC-H slices) picrotoxin reversibly induced spontaneous rhythmic paroxysmal discharges (supplemental Fig. 2*B*, available at www.jneurosci.org as supplemental material).

Patterns of intrinsic firing activity of developing mEC neurons

We further investigated the intrinsic firing pattern of mEC LIII principal neurons at P5–P13 with the goal to detect spontaneously active cells. Recordings were obtained during blockade of glutamatergic and GABAergic neurotransmission with a drug mixture consisting of either a mixture of kynurenic acid (2 mM) and picrotoxin (100 μM), or a mixture of APV (60 μM), CNQX (30 μM), and picrotoxin (100 μM). Under these conditions, we identified three distinct patterns of intrinsic firing behavior.

We found that a fraction of immature LIII neurons spontaneously generated prolonged (~2–20 s) voltage-dependent intrinsic bursting activity, as illustrated in Figure 3*Aa*. At P5–P7, in standard bicarbonate-based ACSF containing 2 mM Ca^{2+} , 25% of the neurons (22 of 88) displayed such a prolonged bursting pattern. This pattern was characterized by a slow periodic (~0.01–0.1 Hz) membrane depolarization (~6 mV) with superimposed spike discharges. Spiking activity during prolonged depolarization was almost regular; however, it occasionally included short-duration (up to ~1 s) bursts. Prolonged bursts of spikes were preceded by a slow regenerative depolarization, which started at approximately −64 mV, and was followed by a small afterhyperpolarization (AHP). Duration of prolonged bursts increased and interburst interval decreased with membrane depolarization; however, the averaged frequency (i.e.,

number of bursts per minute) did not significantly change with membrane depolarization (*n* = 9) (supplemental Fig. 3*A*, available at www.jneurosci.org as supplemental material). Indeed, negative current injection (membrane hyperpolarization beyond approximately −70 mV) led to silencing (Fig. 3*Aa*); in initially silent neurons, depolarizing current injection to approximately −60 mV produced bursting activity. The resting membrane potential (RMP) of the neurons was −61.8 ± 3 mV (*n* = 12). The histogram of membrane potential distribution of prolonged-bursting neurons showed two distinct peaks reflecting the two states of membrane potential (Fig. 3*Aa*, right). The quantitative parameters of prolonged intrinsic bursts are summarized in Table 2. Prolonged bursting activity recorded during intact neurotransmission displayed similar characteristics but was accompanied by brief synaptically mediated bursts that remained after negative current injection to voltage levels below approximately −68 mV (*n* = 4) (supplemental Fig. 3*B*, available at www.jneurosci.org as supplemental material). Since whole-cell recordings accompanied by intracellular dialysis with the pipette filling solution could modify firing pattern, we used the cell-attached configuration to confirm bursting behavior of intact mEC neurons. Robust prolonged bursting activity similar to the whole-cell recordings was detected in the cell-attached configuration (*n* = 23) (supplemental Fig. 3*C*, supplemental Table 1, available at www.jneurosci.org as supplemental material), indicating that this burst firing is a physiological firing mode of developing mEC LIII neurons.

The majority of LIII neurons (72%, 63 of 88 at P5–P7 in 2 mM $[Ca^{2+}]_o$) displayed a nonbursting (regular) pattern of activity, as illustrated in Figure 3*Ab* [i.e., neurons were silent at the RMP and showed regular firing activity following membrane depolarization; RMP: −60.7 ± 3.7 mV (*n* = 25); input resistance (IR): 1.2 ± 0.2 GOhm (*n* = 4)]. The remaining 3% of neurons (*n* = 3) showed a spontaneous activity that was classified as “short bursting” (Fig. 3*Ac*). This pattern was characterized by the exclusive presence of short-duration (< 2 s) suprathreshold membrane depolarization, including a complex of two to four fast spikes (RMP: −60 ± 3.1 mV; IR: 1.1 ± 0.2 GOhm). The histogram of membrane potential displayed one peak distribution (Fig. 3*Ac*, right).

Thus, at P5–P7, in ACSF containing 2 mM Ca^{2+} the proportions of neurons with prolonged bursting, regular firing, and short bursting activity were 25, 72, and 3%, respectively. However, recordings in 1 mM $[Ca^{2+}]_o$ resulted in an increased fraction of neurons with prolonged bursting to 53% (49 of 92 neurons) and with short bursting to 24% (*n* = 22). At the same

Figure 4. I_{CAN} underlies the prolonged intrinsic bursts. **A**, Prevention of prolonged bursting activity after blockade of Ca^{2+} channels with cadmium (Cd^{2+} , 100 μM). Right, Part of the trace marked on the left-hand side shown at an expanded time scale. Note that short bursts of spikes are still present after positive current injection in the presence of Cd^{2+} (indicated by gray arrowhead). **B**, Nifedipine (50 μM) strongly suppressed prolonged bursts. **C**, Intracellular application of the calcium chelator BAPTA (30 min) abolishes prolonged bursts. Current injection is indicated under right-hand trace. **D**, Burst duration and number of spikes per burst under control conditions and in the presence of 50–100 μM nifedipine ($n = 7$) or after buffering intracellular Ca^{2+} with BAPTA ($n = 8$). Averaged data are given as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. **E**, FFA (50 μM) blocks prolonged intrinsic bursting activity. All recordings were made in the presence of kynurenic acid and picrotoxin.

time, we observed a reduction in the amount of the regularly firing cells to 23% ($n = 21$). These results indicate that depending on $[\text{Ca}^{2+}]_o$, some neurons may switch from one firing mode to the other. Indeed, as illustrated in Figure 3*Ba*, we found that the regular firing pattern observed in 2 mM $[\text{Ca}^{2+}]_o$ can switch to the prolonged bursting activity after a reduction of $[\text{Ca}^{2+}]_o$ to 1 mM ($n = 3$ of 7 tested neurons). Nevertheless, four of seven neurons displayed prolonged bursting activity in both tested $[\text{Ca}^{2+}]_o$ concentrations (Fig. 3*Bb*). On average, lowering $[\text{Ca}^{2+}]_o$ to 1 mM did not affect the occurrence of bursts and burst duration. However, reduction of $[\text{Ca}^{2+}]_o$ significantly increased the burst amplitude as well as spike frequency within bursts (Table 2). The amount of neurons generating the prolonged bursting pattern peaked around P8–P10, and then at P11–P13 strongly decreased. In 1 mM $[\text{Ca}^{2+}]_o$, percentages of neurons showing prolonged bursting, short bursting, and regular firing activity, were 81, 5, and

14%, respectively, at P8–P10 ($n = 44$), and 29, 0, and 71%, respectively, at P11–P13 ($n = 49$) (Fig. 3*C*). Significant alterations in parameters of prolonged intrinsic bursting were observed at P8–P10 versus P5–P7, but not at P11–P13 versus P8–P10 (Table 2). Basically, the occurrence of bursts increased and burst duration markedly decreased during the first two postnatal weeks (tested from P5). Similar alterations were also observed in the cell-attached patch recordings (supplemental Table 1, available at www.jneurosci.org as supplemental material). At P8–P10 and P11–P13, membrane hyperpolarization beyond approximately -70 mV always led to silencing.

The prolonged intrinsic bursting activity was no longer detected in the third postnatal week [tested in 1 mM $[\text{Ca}^{2+}]_o$ by using whole-cell ($n = 6$) and sharp microelectrode ($n = 10$) recordings]. Thus, the described phenomenon may selectively contribute to early postnatal development.

Ionic mechanisms of prolonged intrinsic bursting activity in developing mEC neurons

We next investigated the mechanisms underlying intrinsic bursting. Knowing that Ca^{2+} -dependent mechanisms are often involved in burst generation, we focused on the possible involvement of the Ca^{2+} -activated nonspecific cationic current (I_{CAN}). To examine the role of extracellular Ca^{2+} influx, we first blocked Ca^{2+} channels with cadmium (Cd^{2+} , 100 μM). This prevented the cells' ability to generate prolonged bursts ($n = 8$) (Fig. 4*A*), suggesting that Ca^{2+} influx associated with spiking is required. Since spiking is expected to lead to Ca^{2+} influx through high-voltage-activated Ca^{2+} channels, we tested the effect of the L-type Ca^{2+} channel blocker nifedipine (10–100 μM). As illustrated in Figure 4, *B* and *D*, nifedipine

(50–100 μM) strongly affected the prolonged bursting activity in all cases tested. Ten micromoles of nifedipine modified regular firing during prolonged bursts into complex spikes ($n = 2$). We also found that high concentrations of intracellular BAPTA, a high-affinity Ca^{2+} chelator, abolished prolonged bursts (Fig. 4*C,D*) (BAPTA vs control; spike frequency within burst: 11.4 ± 5.6 Hz vs 14.6 ± 4.5 Hz, $p < 0.05$; number of bursts per minute: 10 ± 2.5 vs 5 ± 2 , $p < 0.02$, $n = 8$). Moreover, prolonged intrinsic bursting activity was completely blocked by FFA (50 μM , $n = 8$) (Fig. 4*E*), a nonselective blocking agent of I_{CAN} (Partridge and Valenzuela, 2000), suggesting a contribution of I_{CAN} to the phenomenon. However, following positive current injections, short bursts of spikes were still observed in the presence of Cd^{2+} or BAPTA (Fig. 4). This could be a result of the persistent Na^+ current (I_{Nap}) which is likely to be involved in the initiation phase of prolonged bursts and possibly in the “up phase” of bursts.

Spikes triggered by I_{Nap} possibly elicit an increase of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) required for the activation of I_{CAN} . Indeed, prolonged bursting activity was completely blocked by TTX ($1 \mu\text{M}$, $n = 4$) (Fig. 5A), an antagonist of both Na^+ currents [I_{Nap} and transient Na^+ current (I_{Nat})]. Furthermore, prolonged bursts were strongly suppressed by riluzole ($5\text{--}10 \mu\text{M}$, $n = 10$) (Fig. 5B), a drug that affects I_{Nap} at far lower concentrations than I_{Nat} [e.g., IC_{50} of 2 vs $50 \mu\text{M}$, respectively (Urbani and Belluzzi, 2000)], pointing to an involvement of the I_{Nap} in triggering and/or sustaining prolonged bursts (riluzole vs control; spike frequency in bursts: $1.9 \pm 0.9 \text{ Hz}$ vs $3.9 \pm 2 \text{ Hz}$, $p < 0.02$; burst amplitude: $8.9 \pm 2.8 \text{ s}$ vs $10.9 \pm 3.7 \text{ s}$, $p = 0.08$, $n = 7$). Moreover, we found that a $0.2\text{--}1 \text{ s}$ suprathreshold current step stimulus was able to elicit a terminated plateau potential in prolonged-bursting ($n = 31$) (Fig. 6A) but not in regularly firing ($n = 11$) or short-bursting ($n = 6$) neurons. The depolarizing plateau potential displayed pronounced activity and voltage dependence (Fig. 6A*b*; supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Thus, increasing the duration or intensity of the trigger pulse as well as the steady depolarization of the membrane potential led to an increase in the duration of the plateau, but never gave rise to a stable state of sustained spiking. In general, a $0.5\text{--}1 \text{ s}$ long spike train at $4\text{--}8 \text{ Hz}$ evoked from a resting level of $\sim 15 \text{ mV}$ or less from spike threshold almost invariably elicited plateau potentials with a duration of $\sim 5 \text{ s}$ at P5–P7. Repetitively ($1\text{--}5 \text{ s}$ after discharges) applied suprathreshold depolarizing pulses revealed refractoriness of the plateau potential ($n = 10$) (Fig. 6A*c*). Recovery from the refractoriness required $\sim 10 \text{ s}$. The basic characteristics of the plateau potential in prolonged-bursting neurons recorded in 1 mM $[\text{Ca}^{2+}]_o$ at different postnatal periods are summarized in Table 3. We also tested whether local synaptic activation could also induce plateau potential in mEC neurons. To eliminate a possible contribution of NMDARs to the plateau, experiments were made in the presence of APV ($30 \mu\text{M}$). As illustrated in Figure 6B, electrical stimulation of the IEC (0.2 ms , $20\text{--}30 \text{ V}$) was sufficient to induce terminated plateau potentials, comparable to those inducing by current step stimulus ($n = 4$). The terminated plateau potential response to current steps was blocked by 30 mM BAPTA applied intracellularly through the patch pipette solution for $\sim 20 \text{ min}$ ($n = 8$) or by $50 \mu\text{M}$ FFA ($n = 5$) (Fig. 7A,B). The plateau was suppressed by $10 \mu\text{M}$ riluzole ($n = 4$) (plateau amplitude: $8.8 \pm 2.3 \text{ mV}$ in riluzole vs $10.5 \pm 0.7 \text{ mV}$ in control) (Fig. 7C). The effects of BAPTA, FFA, and riluzole on the plateau are comparable to their effects on spontaneous prolonged bursting activity. These results suggest that both I_{CAN} and I_{Nap} are involved in the generation of plateau potentials as well as in “up phase” of prolonged bursts. Importantly, elevation of $[\text{Ca}^{2+}]_o$ from 1 to 2 mM resulted in a switch from prolonged bursting activity to regular firing and was also accompanied by the disappearance of the plateau potential ($n = 4$) (Fig. 7D).

Figure 5. Na^+ currents are involved in the generation of prolonged intrinsic bursting activity. **A**, TTX ($1 \mu\text{M}$) completely blocks spontaneous bursting activity. **B**, Riluzole ($10 \mu\text{M}$) strongly suppress prolonged bursting activity. The corresponding histogram of membrane potential distribution calculated for 50 s of recording (bin size, 1 mV) shows two distinct peaks in control conditions and one peak in the presence of riluzole (bottom). All recordings were obtained in the presence of kynurenic acid and picrotoxin.

In the thalamus, the mechanism underlying the generation of the “up state” in the slow oscillation critically relies on the window component of the low-voltage-activated Ca^{2+} current (T type) (Crunelli et al., 2005; Blethyn et al., 2006). Moreover, Ca^{2+} entry through T-type channels may activate I_{CAN} (Bal and McCormick, 1993). To investigate a possible contribution of T-type Ca^{2+} channels in the generation of prolonged intrinsic bursts, we performed additional experiments in the presence of $100 \mu\text{M}$ Ni^{2+} , a concentration that preferentially blocks T-type Ca^{2+} channels. Addition of Ni^{2+} did not affect prolonged bursting activity of mEC LIII neurons ($n = 3$) (supplemental Table 2, available at www.jneurosci.org as supplemental material). To analyze whether Ca^{2+} -activated K^+ currents (I_{AHP}) play a role in the termination of prolonged bursts, TEA (1 mM) was applied at a concentration that blocks the large-conductance Ca^{2+} -dependent K^+ current (BK current). As illustrated in Figure 8A, TEA elicited a strong prolongation of burst duration with a simultaneous increase in burst amplitude (supplemental Table 3, available at www.jneurosci.org as supplemental material). Duration and amplitude of plateau potentials triggered by depolarizing current steps were also enhanced in the presence of TEA (Fig. 8B; also supplemental Table 4, available at www.jneurosci.org as supplemental material). We also tested neurons in the presence of the group I and II metabotropic glutamate receptor (mGluR) antagonist E4CPG to exclude a possible early mGluR-dependent firing (Yoshida et al., 2008). Bath application of the E4CPG ($200 \mu\text{M}$) did not abolish the prolonged intrinsic bursting activity of immature mEC LIII neurons ($n = 4$). The prolonged intrinsic bursting activity in mEC LIII neurons was also observed in the presence of atropine ($10 \mu\text{M}$, $n = 4$), indicating that cholinergic

Figure 6. Plateau potential in immature mEC LIII neurons. **Aa**, Spontaneous intrinsic bursting activity under control conditions. **Ab**, In the same neuron a short (1 s) suprathreshold current step stimulus is sufficient to elicit a terminated plateau potential. The bottom trace shows responses to equal current steps after membrane hyperpolarization. **Ac**, Repetitively applied suprathreshold depolarizing pulse reveals refractoriness of the plateau potential. Recordings were made in 2 mM $[Ca^{2+}]_o$ in the presence of kynurenic acid and picrotoxin. **B**, Plateau potential induced by current step stimulus (left) and by electrical stimulation (right) in the same neuron. Stimulation of the IEC (0.2 ms, 30 V) is indicated by the arrowhead. Recording was made in 1 mM $[Ca^{2+}]_o$ in the presence of APV (30 μ M).

muscarinic-dependent spiking (Tahvildari et al., 2008) does not underlie this phenomenon.

Effects of lowering $[Ca^{2+}]_o$ and inhibitors of prolonged intrinsic bursts on the periodic spontaneous field activity in the developing mEC

We further investigated the potential contribution of intrinsic bursting to the periodic spontaneous field events. The above results had shown that lowering $[Ca^{2+}]_o$ increased the tendency of mEC LIII neurons to generate bursts. We next examined whether lowering of $[Ca^{2+}]_o$ from 1.6 to 1 mM affects spontaneous fp activity in the mEC.

No obvious alterations in fp activity after lowering of $[Ca^{2+}]_o$ were identified in slices at P1–P4. Periodic spontaneous fp activity was not identified in 11 of 22 tested slices (50%). In six slices (27%) individual negative fp deflections (range: 0.7 ± 0.2 s, 0.06 ± 0.03 mV, ~ 2 –20 deflections/10 min) accompanied by

Table 3. Quantitative parameters of the plateau potential at different postnatal periods

	1 mM $[Ca^{2+}]_o$		
Parameter (mEC LIII)	P5–P7 (<i>n</i> = 10)	Versus P8–P10 (<i>n</i> = 6)	Versus P11–P13 (<i>n</i> = 5)
Plateau duration (s)	4.8 ± 1.8 *	2.8 ± 1.7 ns	2.7 ± 0.7
Plateau amplitude (mV)	9.2 ± 2.3 ns	11.6 ± 3.7 ns	11.3 ± 2.5
Spike frequency within plateau (Hz)	4.4 ± 1.3 **	14.8 ± 10.6 ns	11.5 ± 0.6
Number of spikes within plateau	21 ± 11 ns	32 ± 17 ns	31 ± 6
AHP amplitude (mV)	3.2 ± 0.9 ns	3.4 ± 1.4 ns	4 ± 1.3
AHP duration (s)	12.6 ± 8.5 ns	6.5 ± 2.4 ns	7 ± 4.6
Recording membrane potential (mV)	-63.5 ± 3.2 ns	-63.1 ± 2.6 ns	-62.8 ± 1.3

Averaged data are given as mean $\pm$ SD. The plateau potential was induced by depolarizing current steps (5–10 pA, 0.2–1 s). Recordings were made in ACSF containing 1 mM Ca^{2+} . iGluRs and GABA_ARs were blocked as detailed in Materials and Methods. Unpaired two-tailed *t* test or Mann–Whitney rank sum test (versus, i.e., left versus right) values are indicated as follows: * *p* < 0.05, ** *p* < 0.01. ns, Not significant.

unit discharges were observed, and in the remaining five slices (23%) only individual unit bursts were detected.

At P5–P7, spontaneous sharp fp events were not significantly changed after reduction of $[Ca^{2+}]_o$ in all six EC–H slices showing selective sharp fp events in control (Fig. 9Aa,Ad). However, lowering of $[Ca^{2+}]_o$ in slices with a selective fp burst activity in control conditions induced a prolongation of fp bursts (*n* = 7) (Fig. 9Aa,Ad). Frequency of the occurrence of fp events was slightly, but not significantly, increased after reduction of $[Ca^{2+}]_o$ to 1 mM (sharp fp events: $111 \pm 14\%$ of control, *n* = 6, *p* = 0.17; fp burst: $133 \pm 41\%$ of control, *n* = 6, *p* = 0.11).

At P8–P10, reduction of $[Ca^{2+}]_o$ to 1 mM reversibly induced a prolongation of fp bursts in slices displaying rhythmic fp bursting under control conditions (*n* = 8) (Fig. 9Ab,Ad). Frequency of fp bursts was not significantly changed in low $[Ca^{2+}]_o$ ($133 \pm 42\%$ of control, *n* = 7, *p* = 0.08). In one slice (P8), showing sharp fp events in the control, lowering $[Ca^{2+}]_o$ strongly and reversibly increased events frequency (FE) and duration (percentage of values in 1.6 mM $[Ca^{2+}]_o$; DE: 141%; PA: 91.5%, FE: 266%) (Fig. 9Ab, top trace).

In five of seven tested slices at P11–P13, lowering of $[Ca^{2+}]_o$ reversibly modified slow-wave rhythmicity into continuous fp fluctuations (~ 20 –40 Hz), going without well distinguishable fp bursts (Fig. 9Ac). In the remaining two slices reduction of $[Ca^{2+}]_o$ elicited expansion of fp bursts (DE: $\sim 150\%$ of control).

To examine whether prolonged intrinsic bursting of immature mEC neurons is essential for the generation of network periodic activity, we tested the effect of FFA (10–50 μ M), a blocker of prolonged bursts, on spontaneous fp events at P5–P7. We found that 50 μ M FFA completely blocked the fp events in all eight tested slices (Fig. 9Ba). In fact, FFA inhibited periodic field activity in a dose-dependent manner. Thus, bath application of 10 μ M FFA already suppressed the occurrence of fp events up to 50% (*n* = 7) and 20 μ M FFA up to 90% (*n* = 7). Thus, we used 50 μ M to provide the full block in further experiments. At higher concentrations (100–200 μ M), FFA has several nonspecific effects, including influence on calcium channels and NMDARs (Wang et al., 2006). Since periodic spontaneous network activity critically depends on iGluR-mediated neurotransmission, we investigated a possible influence of 50 μ M FFA on the excitatory postsynaptic field potential (fEPSP) (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). We found that FFA (bath application for 40–60 min) only slightly affected AMPA/kainate receptor- and NMDAR-mediated components

of synaptic responses (percentage of the control amplitude; antidromic response: $102 \pm 11\%$, $p = 0.73$; AMPA/kainate: $122 \pm 8\%$, $p = 0.004$; NMDA: $90 \pm 4\%$, $p = 0.006$, $n = 5$) (for details, see Materials and Methods). In addition, fp events evoked by electrical stimulations were completely blocked by FFA, and a further increase of stimulus intensity (tested up to 40 V) causes an enhancement in synaptic responses but was unable to reinstate fp events ($n = 8$) (supplemental Fig. 5C, available at www.jneurosci.org as supplemental material), suggesting that the effect of FFA on spontaneous fp events was not due to a suppression of synaptic transmission.

However, I_{CAN} might contribute to the depolarization of EC neurons, and thus blockade of I_{CAN} by FFA can decrease the general level of tonic excitation that is essential for the promotion of the periodic spontaneous network activity. We therefore examined whether FFA could be also efficient after cellular depolarization caused by elevation of $[K^+]_o$ to 8 mM. Indeed, we found that FFA suppressed periodic spontaneous fp activity to $\sim 90\%$, even in high $[K^+]_o$ ($n = 10$) (Fig. 9Bb), suggesting that the effect of FFA was not through its action on the tonic depolarization but probably was related to the blockade of prolonged intrinsic bursts. Riluzole [$10 \mu M$ ($n = 6$), five slices tested in 5.5 mM $[K^+]_o$] also blocked spontaneous fp events, though field synaptic response was strongly reduced ($\sim 80\%$). Finally, we found that 50 μM FFA (bath application for 40–60 min) modulated spontaneous slow-wave network rhythmicity at P11–P13 (percentage of control values; DE: $41 \pm 15\%$, $p < 0.005$; PA: $121 \pm 51\%$, $p = 0.25$; FE: $180 \pm 69\%$, $p < 0.01$; $n = 9$) (Fig. 9Bc). The above experiments suggest that prolonged intrinsic bursting activity may contribute to the generation of network periodic events in the immature mEC.

Discussion

In this study, we investigated the early spontaneous fp activity and intrinsic firing pattern of developing mEC LIII principal neurons. We identified three distinct patterns of intrinsic firing behavior of immature neurons: prolonged bursting, short bursting, and regular firing activity. We found that $[Ca^{2+}]_o$ modulates intrinsic firing mode at a range of 1–2 mM. We propose that I_{CAN} and I_{Nap} underlie prolonged bursts and that I_{AHP} (BK current) is involved in burst termination. We further suggest that prolonged intrinsic bursting activity may participate in the generation of fp events.

Developmental profile of periodic spontaneous field activity in the immature mEC

Spontaneous fp activity in rat entorhinal cortical slices is characterized by its weak expression around birth (P1–P4) and periodic

Figure 7. Ionic mechanism of terminated plateau potential in immature mEC LIII neurons. **A, B,** The plateau potential response to current steps is completely blocked by buffering intracellular Ca^{2+} with 30 mM BAPTA (**A**) or by bath application of 50 μM FFA (**B**). **C,** The plateau potential is suppressed by 10 μM riluzole (indicated by the arrowhead). Recordings were made in ACSF containing 1 mM Ca^{2+} . **D,** Increase of $[Ca^{2+}]_o$ from 1 to 2 mM that resulted in a switch from prolonged bursting activity to regular firing is accompanied by the disappearance of the plateau potential. All recordings were obtained in the presence of kynurenic acid and picrotoxin.

fp events at P5–P7 and is followed by slow-wave network rhythmicity. The latter becomes dominant at P11–P13 and during the juvenile period. In general, spontaneous fp activity is markedly increased during the first two postnatal weeks. Interestingly, quite similar activity patterns (spontaneous periodic events and SWOs) were reported in the mEC LIII of adult rodents *in vitro* (Dickson et al., 2003; Cunningham et al., 2006; Gnatkovsky et al., 2007); however, the underlying mechanisms could be different. Slow-wave activity at P11–P13 possibly represents a pattern observed during sleep and anesthesia (Steriade, 2006). cENOs and cGDPs are synapse-mediated distinct early network patterns, characterized by different spatiotemporal dynamics both in electrical and optical recordings (Allène et al., 2008). We investigated spontaneous network activity in slices by using only fp record-

Figure 8. Ca^{2+} -activated K^+ currents (BK currents) play a role in the termination of prolonged intrinsic bursting activity. **A**, Prolonged bursting activity in control conditions and after the addition of TEA (1 mM). TEA prolongs burst duration and simultaneously increases the firing frequency during bursts. **B**, Duration and firing frequency of the plateau potential triggered by a depolarizing current step are also significantly enhanced in the presence of TEA. All recordings were made in the presence of kynurenic acid and picrotoxin.

ings; thus, it is difficult to compare it to the described patterns precisely. Periodic fp activity in the immature mEC is mediated by iGluRs, similar to cENOs, and different to cSPAs or other early gap junction synchronized cortical oscillations (Sun and Luhmann, 2007). The duration of fp bursts is also comparable to the duration of cENOs measured by fp recordings (~ 3 s). However, in contrast to the somatosensory cortex, in which expression of cENOs peaks around birth (P0–P3) and vanishes when cGDPs dominate the network (P6–P8), fp activity in EC slices is weak at P1–P4, and iGluRs-mediated fp bursts initially appear around P5. Such time differences could reflect the different functions of these two cortical structures. The mEC is involved in spatial representation and navigation (Moser et al., 2008) that, in comparison with sensory representation, might be less essential at very early postnatal stages. We did not observe a principal pattern corresponding to GABA-driven cGDPs (Allène et al., 2008), and a selective blockade of GABA_A receptors always elicited paroxysmal field discharges. Thus, iGluR-mediated neurotransmission seems to dominate in the generation of early network activity in the mEC. However, our results do not exclude the presence of GABA-driven activity in the immature mEC, since slow-wave pattern can suppress/hide it.

Ionic mechanisms of prolonged intrinsic bursting activity

Importantly, a fraction of developing mEC LIII neurons spontaneously generate prolonged Ca^{2+} - and voltage-dependent intrinsic bursting activity. Intrinsic mechanisms for burst generation have been demonstrated in a variety of neurons (Crunelli et al., 2005; Bal and McCormick, 1993; Del Negro et al., 2005), including the juvenile visual cortex (Le Bon-Jego and Yuste, 2007) and hippocampus (Su et al., 2001; Sipilä et al., 2005). Based on the physiological and pharmacological properties of prolonged bursts, we suggest the following cascade of currents underlying the prolonged bursting activity in developing mEC LIII neurons: (1) slow regenerative depolarization leading to intrinsic bursts is driven by I_{Nap} and inactivating Ca^{2+} -dependent K^+

currents, but not by low-threshold T-type Ca^{2+} currents; (2) membrane depolarization (and subsequent Na^+ -dependent spikes) induces activation of high-voltage-activated Ca^{2+} channels (nifedipine-sensitive L-type Ca^{2+} channel), resulting in I_{CAN} activation; (3) both I_{CAN} and I_{Nap} are involved in the generation of plateau phase of prolonged bursts; (4) the further increase in $[\text{Ca}^{2+}]_i$ occurring during the spiking activates TEA-sensitive and probably other Ca^{2+} -dependent K^+ currents that elicit burst termination and the following refractory period. In prolonged-bursting neurons, a suprathreshold current step elicits a terminated plateau potential, which is comparable in duration with spontaneous prolonged bursts. Pharmacological properties suggest identical ionic mechanisms for both events, except initiation phase (1).

Our observation that blockade of T-type Ca^{2+} current did not promote prolonged bursting is in agreement with the fact that in adult LIII pyramidal neurons, subthreshold depolarization to approximately -53 mV does not evoke intracellular Ca^{2+} -accumulation (Gloveli et al., 1999). In mEC LIII, selective blockade of the M current by linopirdine or XE991 switches firing pattern from tonic to bursting mode (Yoshida and Alonso, 2007). We do not exclude the possibility that in immature mEC LIII neurons weakness of M current may contribute to prolonged burst firing. Moreover, Kv7/M channel blocker linopirdine has only a minor effect on neonatal, in contrast to juvenile CA3 pyramidal neurons (Safiulina et al., 2008).

Role of prolonged intrinsic bursting in the generation of periodic spontaneous field events

A central role of intrinsic firing in the generation of network events has been demonstrated in various neuronal structures, including the immature retina (Zheng et al., 2006), and changes in the intrinsic firing properties of individual neurons have important roles in altering circuit behavior (Destexhe and Marder, 2004). Sipilä and colleagues (2005; 2006) have hypothesized that intrinsic bursting of CA3 pyramidal neurons drives and shapes hippocampal GDPs, although GABA- and glutamate-mediated transmissions promote their generation. Our results suggest that prolonged intrinsic bursting of principal neurons may contribute to the generation of periodic spontaneous fp events in the immature mEC. We show that fp events in the mEC occur hippocampus-independently and require selective activation of iGluRs. Both fp events and prolonged firing are inducible by synaptic activation. Elevation of $[\text{K}^+]_o$ as well as lowering $[\text{Ca}^{2+}]_o$ resulted in an increase in prolonged intrinsic bursting leading to an appearance of fp bursts or to a significant increase of its duration. Finally, FFA (a blocker of prolonged intrinsic bursts) completely prevents spontaneous fp events at P5–P7, without affecting tonic depolarization or synaptic neurotransmission. However, in view of the nonspecificity of FFA, this point needs further investigation.

Figure 9. Effects of lowering $[Ca^{2+}]_o$ and FFA on periodic spontaneous field activity in the developing mEC. **A**, Spontaneous fp

$[Ca^{2+}]_o$ dependency and functional implications of prolonged bursting

The pattern of intrinsic firing activity of immature mEC LIII neurons displays a significant $[Ca^{2+}]_o$ dependence: lowering $[Ca^{2+}]_o$ increases, whereas elevating $[Ca^{2+}]_o$ decreases the tendency to generate bursts. A similar tendency was described for adult hippocampal CA1 pyramidal neurons (Su et al., 2001). The burst-firing of CA1 neurons is I_{NaP} -dependent and thus, lowering $[Ca^{2+}]_o$ to 1 mM induced intrinsic bursting preferentially by increasing I_{NaP} [i.e., by shifting voltage dependence of I_{NaP} activation to more negative potentials (Yue et al., 2005)]. We suggest that the $[Ca^{2+}]_o$ -dependent shift of I_{NaP} -activation in mEC LIII neurons also plays a central role in the augmentation of prolonged bursts or in the transformation of regular firing to prolonged-bursting mode. Additionally, low $[Ca^{2+}]_o$ could influence the balance between depolarizing and hyperpolarizing currents (e.g., I_{CAN}/I_{AHP}) that might be essential for these transformations.

The perforant path (or temporoammonic path) provides direct input from EC LIII to the apical dendritic tuft of hippocampal CA1 pyramidal neurons (Witter and Amaral, 2004). This suggests that neuronal activity of immature mEC LIII neurons contributes to hippocampal development. Thus, a direct excitatory pathway from LIII is critical for the establishment and maintenance of distal dendritic enrichment of a HCN1 channel in CA1 pyramidal neurons (Shin and Chetkovich, 2007). Interestingly, modeling studies have revealed that strong per-

activity in the immature mEC before and after reduction of $[Ca^{2+}]_o$ to 1 mM. **Aa**, At P5–P7, lowering of $[Ca^{2+}]_o$ does not change sharp fp events (top); however, it significantly increases the duration of fp bursts (bottom). **Ab**, Top, Specimen trace of an fp recording of spontaneous sharp fp events in a P8 slice under control conditions and after reduction of $[Ca^{2+}]_o$. Note that lowering of $[Ca^{2+}]_o$ strongly increases event frequency. **Ab**, Bottom, Characteristic prolongation of fp bursts at P8–P10 caused by reduction of $[Ca^{2+}]_o$. **Ac**, At P11–P13, decreasing $[Ca^{2+}]_o$ modifies slow-wave rhythmicity into continuous fp fluctuations. Parts of the traces noted by horizontal bars are shown below in the expanded time scale. **Ad**, Bar diagrams representing the mean durations and peak amplitudes of sharp fp events and fp bursts in 1.6 and 1 mM $[Ca^{2+}]_o$. Error bars indicate SD. * $p < 0.05$. ns, Not significant. **B**, Effect of FFA on spontaneous fp activity at P5–P7 and P11–P13. **Ba**, Periodic spontaneous fp events are fully blocked in the presence of 20 μ M FFA (sample at P7). **Bb**, Spontaneous fp activity increases during enhanced tonic excitation following elevation of $[K^+]_o$ and is strongly decreased after adding 50 μ M FFA (sample trace at P5). **Bc**, FFA (50 μ M) modulates spontaneous slow-wave network rhythmicity at P11–P13.

forant path activation is required to induce dendritic spikes in the distal apical dendrite that could propagate to the soma of CA1 pyramidal neurons and trigger an action potential (Jarsky et al., 2005), suggesting that distal CA1 dendritic signals propagate more reliably during EC LIII bursts. We detected intrinsic bursting activity only in early postnatal, but not in adult mEC LIII neurons (tested in 2 and 1 mM $[Ca^{2+}]_o$). This confirms previous *in vitro* observations showing that mEC LIII pyramidal cells of adult rats are regularly spiking neurons that do not discharge bursts of action potentials (Dickson et al., 1997; Gloveli et al., 1997; Yoshida and Alonso, 2007). However, in brain slices of macaque monkeys, it has been shown that 19% of mEC LIII neurons display intrinsic bursting (Buckmaster et al., 2004) (tested in 2 mM $[Ca^{2+}]_o$).

Prolonged intrinsic bursting activity is a feature of developing but not mature rat mEC LIII neurons. In contrast to pacemaker-like neuronal activity described in the juvenile mouse visual cortex (Le Bon-Jego and Yuste, 2007), prolonged intrinsic bursting activity is not observed at the third postnatal week. Importantly, the intrinsic firing pattern is not an invariable permanent attribute of developing mEC LIII neurons. However, our results suggest that neurons which are already able to convert from immature prolonged bursting to the mature regular firing pattern by 2 mM $[Ca^{2+}]_o$ are more mature than neurons showing prolonged bursting activity at both $[Ca^{2+}]_o$ concentrations. Moreover, the switch to a mature regular firing mode could be due to developmental changes in the I_{CAN}/I_{AHP} ratio (our unpublished observations), such as enhancement of I_{AHP} (Kang et al., 1996) or reduction of I_{CAN} . In mature mEC neurons, I_{CAN} underlies persistent firing (Klink and Alonso, 1997; Egorov et al., 2002; Al-Yahya et al., 2003; Fransén et al., 2006). However, I_{CAN} -mediated persistent activity requires activation of muscarinic-cholinergic receptors or mGluRs, and the postnatal increase in neurons endowed with persistent firing properties in mEC was found to parallel the development of the cholinergic system (Reboreda et al., 2007). Thus, the decrease of I_{CAN} might result from its functional switch from spontaneous Ca^{2+} -dependent activation at an early developmental stage to a muscarinic receptor/mGluR-mediated activation at a mature stage. To conclude, we suggest that prolonged intrinsic bursting of immature mEC LIII neurons could play an important role in cortical and hippocampal development.

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