Behavioral/Systems/Cognitive

Synchronous Bursts of Neuronal Activity in the Developing Hippocampus: Modulation by Active Sleep and Association with Emerging Gamma and Theta Rhythms

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The neonatal hippocampus exhibits regularly recurring waves of synchronized neuronal activity *in vitro*. Because active sleep (AS), characterized by bursts of phasic motor activity in the form of myoclonic twitching, may provide conditions that are conducive to activity-dependent development of hippocampal circuits, we hypothesized that the waves of synchronous neuronal activity that have been observed *in vitro* would be associated with AS-related twitching. Using unanesthetized 1- to 12-d-old rats, we report here that the majority of neurons in CA1 and the dentate gyrus (DG) are significantly more active during AS than during either quiet sleep or wakefulness. Neuronal activity typically occurs in phasic bursts, during which most neurons are significantly cross-correlated both within and across the CA1 and DG fields. All AS-active neurons increase their firing rates during periods of myoclonic twitching of the limbs, and a subset of these neurons exhibit a burst of activity immediately after limb twitches, suggesting that the twitches themselves provide sensory feedback to the infant hippocampus, as occurs in the infant spinal cord and neocortex. Finally, the synchronous bursts of neuronal activity are coupled to the emergence of the AS-related hippocampal gamma rhythm during the first postnatal week, as well as the emergence of the AS-related theta rhythm during the second postnatal week. We hypothesize that the phasic motor events of active sleep provide the developing hippocampus with discrete sensory stimulation that contributes to the development and refinement of hippocampal circuits as well as the development of synchronized interactions between hippocampus and neocortex.

Key words: **myoclonic twitching; CA1; dentate gyrus; gamma rhythm; theta rhythm; development**

Introduction

The predominant behavioral state during the neonatal period in rats is active sleep (AS), which is characterized behaviorally by twitches of the limbs, head, eyes, and tail (Gramsbergen et al., 1970; Jouvet-Mounier et al., 1970; Karlsson and Blumberg, 2002; Seelke et al., 2005). These twitches, which are expressed as phasic bursts of activity in multiple muscle groups with interposed periods of silence, are produced by neurons within the mesopontine region and spinal cord (Blumberg and Lucas, 1994; Kreider and Blumberg, 2000; Karlsson et al., 2005). Importantly, the disproportionate amount of time spent in AS during infancy has led researchers to hypothesize that it facilitates brain development, particularly within neocortical and hippocampal networks (Roffwarg et al., 1966; Mirmiran, 1995; Blumberg and Lucas, 1996; Corner et al., 2002).

The *in vitro* firing patterns of hippocampal and neocortical neurons during the early postnatal period differ qualitatively from those seen in adults, appearing in regularly recurring waves of synchronized activity (Leinekugel et al., 1997; Garaschuk et al., 1998, 2000; Crépel et al., 2007). Such synchronous patterns are thought to facilitate activity-dependent development (Katz and Shatz, 1996; Feller, 1999; O'Donovan, 1999). In the first study describing the firing patterns of neonatal CA1 neurons *in vivo* (Leinekugel et al., 2002), the synchronous bursts previously identified *in vitro* were observed, but behavioral state was not quantitatively assessed. Therefore, in the present study, we examined whether sleep, and, in particular, the phasic motor activity of AS, modulates the temporal patterning of unit and field activity in the infant hippocampus.

We recorded the activity of neurons in CA1 and dentate gyrus (DG) of unanesthetized head-fixed rats on postnatal day 1 (P1)–P12. We found that the majority of neurons in the infant hippocampus were significantly more active during periods of AS-related twitching than during quiet sleep (QS) or wakefulness. Moreover, a subset of these neurons exhibited a burst of activity immediately after limb twitches, suggesting that the twitches themselves provide sensory feedback to the developing hippocampus, as occurs in the neonatal spinal cord and neocortex (Petersson et al., 2003; Khazipov et al., 2004). With age, the relationship between neuronal activity and twitching remained stable as gamma, and then theta activity emerged sequentially during AS. This sequence ultimately resulted in the appearance of the combined rhythm that is characteristic of adult hippocampal activity, in which gamma-frequency oscillations are modulated by

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Table 1. Number of CA1 and DG units recorded in each age group

Age group	Number of subjects	CA1 ($n = 74$)	DG ($n = 40$)	Total
P1–P5	5	14	5	19
P6	4	13	5	18
P7	7	23	13	36
P8	4	10	6	16
P9–P10	3	7	6	13
P11–P12	5	7	5	12
				114

theta-frequency oscillations. We conclude that the recurring synchronous bursts of neuronal activity observed *in vitro* are, in the intact animal, modulated by the AS state. Myoclonic twitching, we suggest, provides sensory feedback that contributes to the development and refinement of hippocampal circuits.

Materials and Methods

All experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23) and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Subjects

Twenty-eight P1–P12 male Sprague Dawley Norway rats (*Rattus norvegicus*) from 23 litters were used (Table 1). Unrelated data from some of the subjects were used in a previous study (Mohns et al., 2007). When littermates were used, they were always tested at different ages. Litters were culled to eight pups on the third day after birth (day of birth = day 0). Mothers and their litters were housed in standard laboratory cages ($48 \times 20 \times 26$ cm) in the animal colony at the University of Iowa, where food and water were available *ad libitum*. All subjects were maintained on a 12 h light/dark schedule with lights on at 7:00 A.M., and all tests were conducted between 12:00 and 5:00 P.M.

Surgery

Under isoflurane anesthesia, the subject's skull was exposed and then bleached, dried, and coated with Vetbond (3M) to add strength. To secure the subject's head during testing, a custom-built stainless-steel apparatus (Karlsson et al., 2005), designed to attach to the ear bar and nose bar holders of a stereotaxic apparatus (David Kopf Instruments), was attached to the outer edges of the pretreated skull using cyanoacrylate adhesive gel. This preparation allowed access to the dorsal hippocampus while minimizing movement. EMG electrodes (50 μm diameter; California Fine Wire) were implanted into the subject's right nuchal muscle and left vastus lateralis muscle to identify behavioral state (Karlsson and Blumberg, 2002; Karlsson et al., 2006). Finally, to inhibit movement and calm the subject, it was wrapped gently in gauze (Corner and Kwee, 1976; Karlsson et al., 2005). The subject recovered from surgery for 1 h in a humidified incubator maintained at thermoneutrality (35°C).

Recording procedure

After recovery from surgery, the subject was transferred to a stereotaxic apparatus, and the skull was leveled in the horizontal plane. After a 1 h acclimation period, a recording probe was inserted ~2 mm posterior to bregma and 1–2 mm lateral to midline. During all experiments, body and brain temperatures were maintained at ~35 and 37°C, respectively. If at any point during the procedure the subject appeared to be in distress, the experiment was terminated.

Recordings were performed using 16-site linear silicon probes (100 μm vertical separation between recording electrodes; NeuroNexus Technologies) lowered into the dorsal CA1–DG axis. An insulated silver wire (Medwire; 0.25 mm diameter) inserted into the cerebellum served as reference electrode. A silicon probe was connected to a unity-gain headstage and digital amplifier (Tucker-Davis Technologies) that amplified (10,000×) and filtered (1–3000 Hz bandpass) the neural signals. A 60 Hz notch filter was applied during all recording sessions. EMG signals were amplified (10,000×) and filtered (300–5000 Hz bandpass) using a differential amplifier (A-M Systems). Neural and EMG signals were sampled at 12.5 kHz using a digital interface (Cambridge Electronic Design) and recorded synchronously to hard disk for off-line analysis. Four consecutive 15 min recordings were made for each subject; the record exhibiting the most stable unit activity and the greatest number of state transitions was selected for analysis.

Data analysis

Identification and analysis of unit activity. In CA1 and/or the DG, four recording channels containing heterogeneous unit activity were selected for analysis (see Fig. 1*A*). Using Spike2 software (Cambridge Electronic Design), each of the selected channels was filtered (300–3000 Hz bandpass) and a digital threshold was then set to extract unit data with a signal-to-noise ratio of at least 2:1. Using the multichannel data, principal component analysis was then used for spike sorting (Abeles and Goldstein, 1977). The sorted units were assigned to groups using graphical cluster cutting. To remove artificial clusters, spike waveforms were inspected, and autocorrelations were constructed for each cluster. A cluster was judged to contain a single unit only if the autocorrelation analysis indicated a refractory period of at least 2 ms.

Correlated neuronal firing both within and between CA1 and DG was assessed by constructing cross-correlograms (or cross-coincidence histograms) for each neuron pair (Eggermont, 1992). The number of coincident spikes in trains A and B was determined within ± 100 ms (in 10 ms bins) of the trigger spike (Wilson and McNaughton, 1994). The expected value (E) of the cross-correlogram, under the assumption of independence of the two spike trains, is equal to the product of the number of spikes in trains A and B, divided by the number of bins in the record (Eggermont, 1992). The value of E is thereby normalized by spike count. The SD of E is then equal to $E^{1/2}$, and we considered the correlation between two neurons to be significant when at least 1 of the 20 bins in the cross-correlogram had a value $\geq E + 3$ SDs ($p < 0.002$).

Identification of behavioral state. For each subject, EMG signals were dichotomized into periods of high tone (indicative of wakefulness) and hypotonia/atonia (indicative of sleep) using the following procedure, as described previously (Karlsson et al., 2004). First, the two EMG records were integrated and rectified. Next, the mean amplitude of five 1 s segments of atonia and the mean amplitude of five 1 s segments of artifact-free high tone were determined for each subject, and the midpoint between the two means was calculated. Bouts of sleep and wakefulness were defined as periods in which muscle tone was below or above, respectively, the midpoint value for at least 1 s. This method establishes bouts of sleep and wakefulness that correspond with behavioral observations (Seelke and Blumberg, 2005).

Sleep bouts were further divided into periods of QS and AS. Whereas QS is characterized by hypotonia/atonia and behavioral quiescence, AS is characterized by the appearance of myoclonic twitches against a background of muscle atonia (Jouvet-Mounier et al., 1970; Karlsson et al., 2005; Seelke and Blumberg, 2008). Twitches are phasic, rapid, independent movements of the limbs and tail, which appear as brief "spikes" in EMG recordings (Karlsson and Blumberg, 2002; Seelke et al., 2005). In the present study, we considered such spikes to be twitches when they exhibited amplitudes that were at least two times the baseline activity level, and occurred during periods of muscle atonia.

To assess the relationship between AS and hippocampal activity, we identified each individual twitch in each record. To do this, we developed an off-line method for detecting twitches and converting them from continuous EMG data into digitally represented events more amenable to statistical analyses. This method also allowed us to assess the precise temporal relationship between individual twitches and unit activity. The two EMG channels were rectified and then smoothed using a time constant of 0.001 s. Using Spike2 software, a positive amplitude threshold for event detection was set at two times the mean level of baseline activity for each channel. Wake-related EMG events and electrical and mechanical artifacts were removed manually. This method allowed us to efficiently and accurately identify all twitches represented in the two EMG records.

Identification of state-dependent neuronal activity. The firing rates of hippocampal neurons during wakefulness, QS, and AS were determined.

To initially assess whether neurons were significantly more active during sleep or wakefulness, their mean firing rates during periods of sleep (AS and QS combined) were compared with their mean firing rates during periods of wakefulness using the Mann–Whitney *U* test. For all tests, α was set at 0.05.

To determine whether recorded neurons were preferentially active during AS, we determined their firing rates during periods of twitching. Unit firing within ±500 ms of each twitch was determined using peristimulus unit histograms (10 ms bins). For each neuron, twitch-triggered activity was compared with a second peristimulus unit histogram that was generated by randomizing the temporal organization of the identified twitches using a custom script written for Spike2. A neuron was considered to be significantly more active during AS than during QS if its twitch-triggered histogram had a peak value that was ≥3 SDs ($p < 0.002$) above the random-triggered (baseline) mean.

Identification of gamma and theta activity. Because the hippocampal gamma and theta rhythms have been reported to first appear during the postnatal period examined here (Leblanc and Bland, 1979; Lahtinen et al., 2001; Karlsson et al., 2006), we sought to determine whether there was a relationship between these rhythms, the behavioral state of the rat, and unit activity. Activity in the gamma (20–100 Hz) and theta (4–14 Hz) frequency ranges was first identified manually in the unfiltered local field potential (LFP) traces. To be selected for analysis, oscillations were required to (1) exhibit an amplitude more than two times the baseline activity, (2) exhibit three complete, continuous cycles, each with clear polarity reversals (180 ± 30°) across stratum pyramidale, and (3) propagate at least 300 μm above (i.e., into stratum oriens) and below (i.e., into the stratum radiatum) the stratum pyramidale (Mohns et al., 2007).

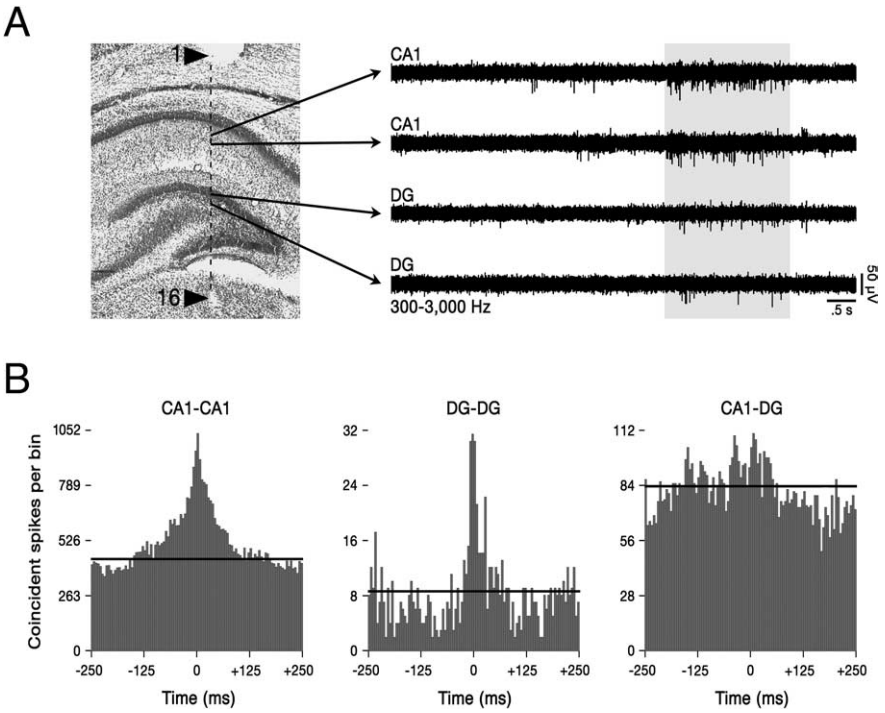

Figure 1. Hippocampal unit activity occurs in synchronous bursts during the early postnatal period. ***A***, Multiunit activity (MUA) in the CA1-DG axis of a representative P6 rat. Left, Histological section showing the location of a 16-site laminar silicon probe. The locations of the shallowest (1) and deepest (16) recording sites are marked with small lesions (arrows). The path of the electrode between these sites is illustrated with a dashed line. Right, Representative MUA from the indicated sites is shown. A bout of correlated MUA is highlighted in gray. ***B***, Correlated firing within and across the CA1 and DG fields, based on the recorded units shown in ***A***. Representative cross-correlograms illustrate coincident spikes for neuron pairs within regions (left, CA1–CA1; middle, DG–DG), as well as pairs across regions (right, CA1–DG). The horizontal line in each cross-correlogram indicates the expected value plus 3 SDs ($p < 0.002$).

Identification of gamma and theta phase modulation of neuronal activity. To assess whether the occurrence of unit activity is modulated by the phase of local oscillations in the infant hippocampus, the following analysis was performed. First, we marked the troughs of the first 100 gamma and theta oscillations in each recording. Next, we constructed separate gamma and theta trough-triggered spike count histograms for each unit (gamma, 2 ms bins; theta, 5 ms bins). We then constructed an additional set of histograms by randomizing each unit's spike train and again triggering by the previously marked troughs; this procedure yielded baseline (or expected) means and their SDs. Finally, for each 2 or 5 ms bin, we defined a significant increase in firing rate as occurring when the firing rate exceeded the baseline mean + 3 SDs ($p < 0.002$).

Next, to examine the phase preferences of units, we generated trough-triggered gamma and theta waveform averages from the LFP channel in which the unit activity was identified. After visual inspection, it was clear that if a unit exhibited a phase preference, that preference was always associated with the peak or trough of the theta oscillation. We compared the number of significant bins during the period centered on the triggered trough (defined as the area below oscillation half-height) to the number of significant bins during the period centered on the preceding peak (defined as the area above oscillation half-height). We concluded that a unit exhibited a trough-related preference when the number of trough-related bins exceeded the number of peak-related bins by ≥300%, or vice versa for a peak-related preference.

Relationship between the gamma and theta rhythms and twitches. To determine the relationship between the gamma rhythm and twitches, periods of identified gamma activity were prepared for analysis by bandpass filtering (20–100 Hz) the LFP traces, and then calculating the root mean square of the filtered data at 10 ms intervals. This provided a continuous measure of gamma power that could be related to behavioral state. The average gamma power for each subject during AS was determined by obtaining the twitch-triggered (±500 ms) average for the prepared traces. To assess the selectivity of coupling between gamma power and twitches, the twitch-triggered average was compared with a random-triggered average; gamma power was considered to be significantly associated with twitching when the average twitch-triggered power was ≥3 SDs ($p < 0.002$) above the random-triggered (baseline) mean. To address the possibility of regional differences in gamma power development, this analysis was performed for hippocampal activity in strata pyramidale, radiatum, and moleculare for each subject.

The relationship between twitching and theta activity was assessed using the procedure just described for gamma activity, except that the LFP signals were bandpass filtered in the theta frequency range (i.e., 4–14 Hz). Analyses of theta power were only performed on subjects beginning at P8, which is when the theta rhythm was first detected in the unfiltered LFP traces.

Detection of hippocampal sharp waves. Sharp waves (SPWs) are associated with both unit activity (Leinekugel et al., 2002) and gamma activity (Mohns et al., 2007) in the developing hippocampus. We therefore investigated the distinctions between SPW-related and AS-related units and oscillations. SPWs were detected by filtering (30 Hz low pass) the wide-band signals and then identifying 40–120 ms events with polarity reversals across stratum pyramidale; these events had positive peaks in stratum oriens and negative peaks in stratum radiatum (O'Keefe and Nadel, 1978; Buzsáki et al., 1983; Suzuki and Smith, 1987). Only events with peak negative amplitudes in stratum radiatum that were at least three times the baseline activity were scored as SPWs (Mohns et al., 2007). Gamma-frequency activity immediately after the SPW was considered a gamma "tail" (Suzuki and Smith, 1988; Bragin et al., 1995a;

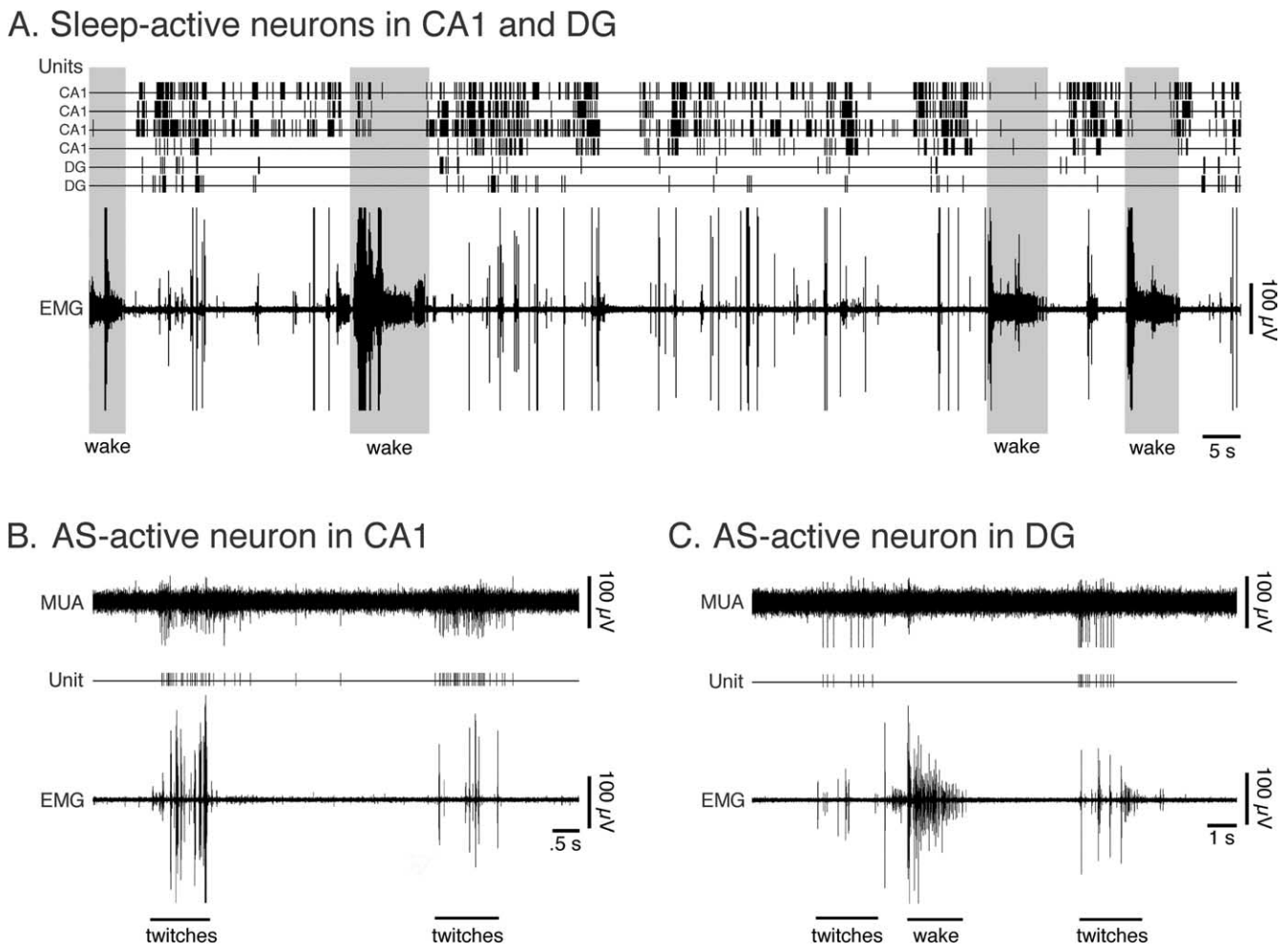

Figure 2. State-dependency of synchronous neuronal activity in the neonatal hippocampus. ***A***, The activity of six simultaneously recorded neurons from a P7 subject illustrates the association between neuronal activity in CA1 and DG and sleep. Nuchal EMG is also shown. Sleep is distinguished by nuchal muscle hypotonia/atonia (with or without myoclonic twitches), whereas wakefulness is distinguished by elevated nuchal muscle tone (gray boxes). ***B***, ***C***, A representative AS-active neuron in CA1 from a P5 rat (***B***) and DG from a P6 rat (***C***). Multiunit activity (MUA; 300–3000 Hz bandpass) detectable at one recording site is shown at the top; the middle trace shows the sorted activity of a single unit from this site. Nuchal EMG is presented below. Note that the units are most active during periods of twitching.

Traub et al., 1996) if it occurred within 100 ms of the SPW negative peak (Mohns et al., 2007).

Histology

When recording was complete, small marking lesions were made at the deepest and shallowest recording sites using a brief application of 50–75 μA anodal current. The subject was then overdosed with an intraperitoneal injection of sodium pentobarbital and transcardially perfused with PBS, followed by a 3% formalin solution. Brains were postfixed for at least 48 h in a formalin–sucrose solution before being sliced in the coronal plane (50 μm sections), mounted, and stained with cresyl violet. Light microscopy was then used to identify the electrode tracks and marking lesions for each subject, and to reconstruct the anatomical locations of the recording sites.

Results

Hippocampal neurons fire in synchronous bursts during the early postnatal period

In total, 114 hippocampal neurons were sampled, including 74 cells in CA1 and 40 cells in the DG (Table 1). Individual unit activity in both CA1 and the DG most often occurred in multispike bursts (Leinekugel et al., 2002). To determine the intraregional and interregional correlation of unit activity, cross-correlograms were constructed for all pairs of simultaneously recorded neurons (86 CA1–CA1 pairs, 31 DG–DG pairs, and 94 CA1–DG pairs) (Fig. 1). Across all ages, the proportion of significantly ($p < 0.002$) cross-correlated neuron pairs was 0.930 for CA1–CA1 pairs, 0.968 for DG–DG pairs, and 0.702 for CA1–DG pairs. Typically, intraregional synchrony created a prominent peak of coincident firing in CA1–CA1 neuron pairs and a narrower peak in DG–DG pairs; CA1–DG neuron pairs were also significantly correlated, but with poorer temporal coordination than that found with the intraregional pairs (Fig. 1). These basic patterns of intraregional and interregional synchrony were found across all ages examined here.

Synchronous bursts of neuronal activity are state dependent

For each neuron sampled, we compared mean firing rates during periods of AS, QS, and wakefulness. These analyses revealed that 95.6% (109 of 114) of the recorded cells exhibited state-dependent firing patterns (see below).

Across all ages, 69.7% (76 of 109) of state-dependent cells were significantly more active during sleep than during wakefulness (sleep-active neurons) (Fig. 2*A*). Of these sleep-active cells, 86.8% (66 of 76) were significantly more active during AS than during QS (AS-active neurons) (Fig. 2*B*,*C*), and the remaining

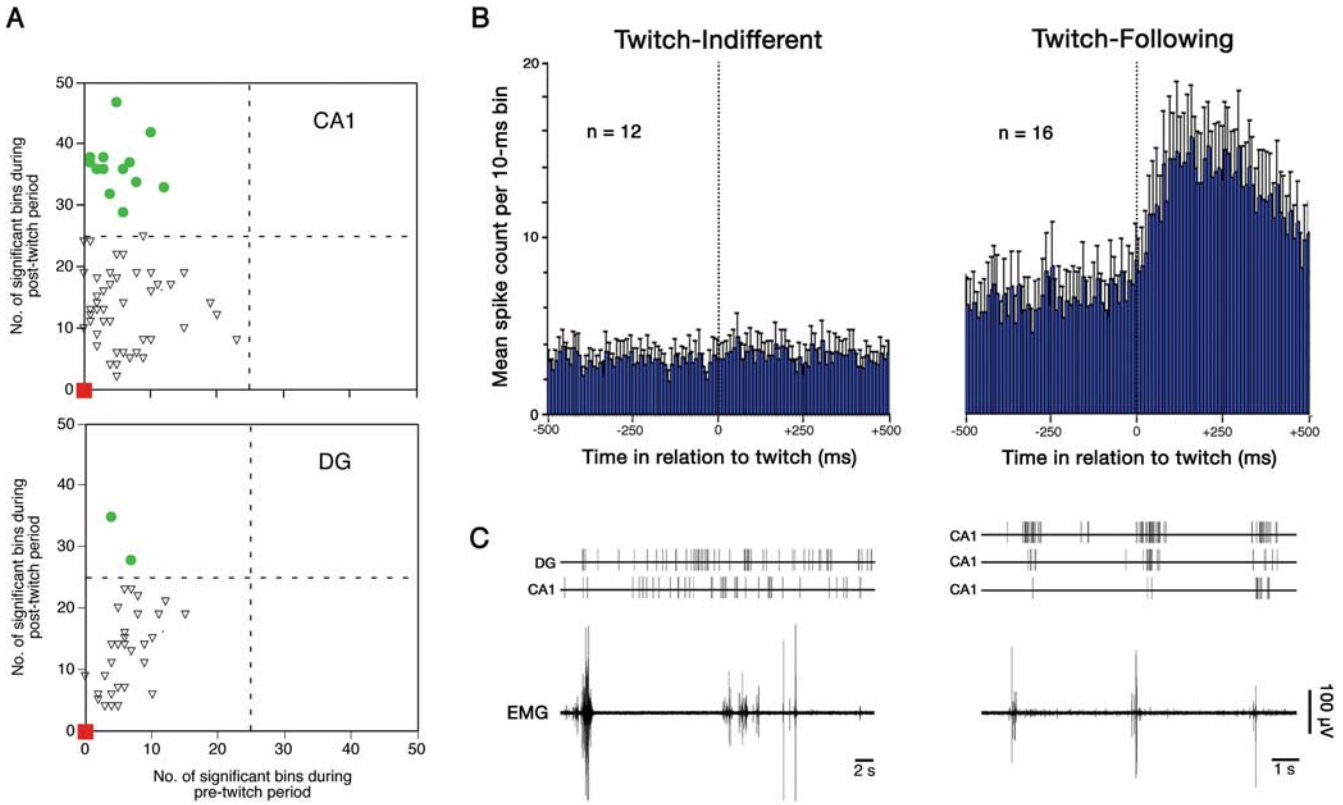

Figure 3. "Twitch-following" hippocampal neurons. ***A***, For all 114 neurons across all P1–P12 subjects, firing rate histograms were constructed (bin size, 10 ms) and the number of significant bins during the 500 ms post-twitch period was plotted against the number of significant bins during the pre-twitch period. These plots are presented separately for CA1 (top) and DG (bottom) neurons. Within each plot, and for purposes of illustration, neurons with no significant increases in firing rate ("twitch-indifferent," red squares; CA1, $n = 7$; DG, $n = 5$) and neurons with significant increases in firing rate immediately after a twitch (twitch-following, green circles; CA1, $n = 14$; DG, $n = 2$) are highlighted. The remaining neurons (open triangles; $n = 86$) exhibited firing patterns that were similar to the twitch-indifferent and twitch-following patterns, as well as a pattern characterized by tonic, high firing rates during the pre-twitch and post-twitch periods. ***B***, Mean twitch-triggered spike count histograms for the twitch-indifferent (left) and twitch-following (right) neurons highlighted in ***A***. Vertical dotted lines at 0 ms indicate the time of twitch occurrence. Data were pooled across CA1 and DG neurons. Twitch-indifferent neurons exhibit no significant association with twitches; twitch-following neurons exhibit an elevated firing rate immediately after a twitch. (mean + SE). ***C***, Representative examples of twitch-indifferent (left; P7 rat) and twitch-following (right; P2 rat) units (above), and corresponding nuchal EMG activity (below). All bursts of activity visible in these EMG records signify twitching activity.

sleep-active cells (10 of 76) showed no preference for AS or QS (AS/QS-indifferent neurons). The remaining 33 of 109 neurons that showed state-dependent firing patterns exhibited significantly increased firing rates during both AS and wakefulness (AS/wake-active neurons).

Thus, a total of 86.8% (99 of 114) of recorded cells exhibited a significant increase in firing rate during AS, with one-third of these cells additionally showing elevated activity during wakefulness. The proportion of cells that were AS active, AS/QS indifferent, and AS/wake active did not differ significantly between CA1 and DG ($X^2 = 0.2$, not significant). In addition, the proportion of cells in each of these categories did not vary across the age groups examined.

A subset of hippocampal neurons increase their firing rate after a twitch

Investigation of the temporal coupling between twitches and unit firing revealed several distinct patterns. To elucidate these patterns, we first determined the number of significant histogram bins (i.e., the number of 10 ms bins >3 SDs above the baseline mean; $p < 0.002$) during the 500 ms periods before and after a twitch. Next, we plotted the number of significant bins during the post-twitch period against the number of bins during the pre-twitch period (Fig. 3*A*). The resulting plot reveals a tendency among many hippocampal neurons to increase their firing rate during the period immediately after a twitch.

Figure 3*A* reveals a continuum of firing patterns for CA1 and DG neurons, from neurons that never significantly increased their firing rate within ±500 ms of a twitch (red squares; designated "twitch indifferent"; $n = 12$ neurons) to neurons that exhibited significant firing rate increases in at least 25 of 50 10 ms bins after a twitch (green circles; designated "twitch following"; $n = 16$ neurons). Mean spike count histograms for neurons within these two extreme categories are presented in Figure 3*B* and representative examples of each are presented in Figure 3*C*. The twitch-following neurons exhibited a distinct burst of activity that peaked ~125 ms after a twitch. Finally, neurons that fell between the two extremes highlighted in Figure 3*A* (open triangles; $n = 86$ neurons) exhibited a continuum of firing patterns: from minimal firing-rate increases, to significant and sustained increases surrounding a twitch, to the twitch-following pattern described above.

Twitch-related population bursts and the emerging gamma rhythm

We observed short bursts of oscillatory activity in the gamma-frequency range as early as P2 that reliably co-occurred with AS-related phasic twitching and simultaneous population bursts.

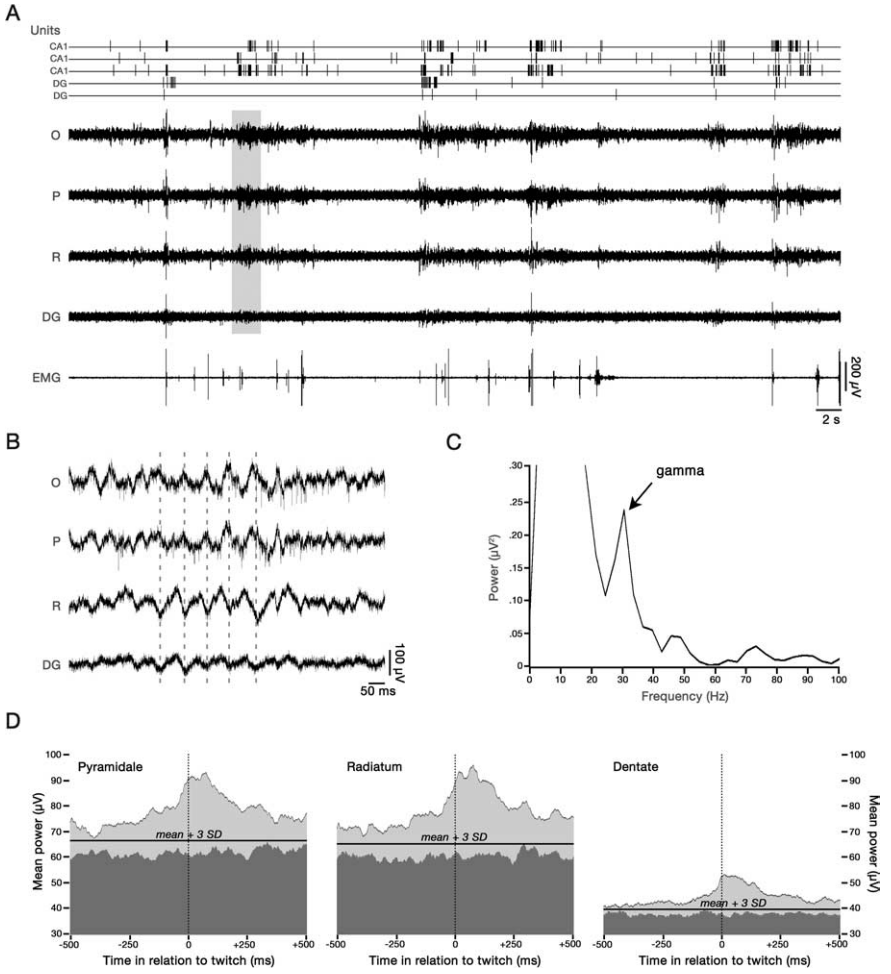

Figure 4. AS-related population bursts and the emerging gamma rhythm. ***A***, Data from a representative P7 subject, including unit activity from five simultaneously recorded neurons; LFP (1–3000 Hz bandpass) activity from strata oriens (O), pyramidale (P), radiatum (R), and DG, and concurrently recorded nuchal EMG activity. All bursts of activity visible in the EMG record signify twitching activity. Note that LFP oscillatory activity emerges during bursts of unit activity, which occur predominantly during periods of AS-related twitches. The segment of data enclosed by the gray box is expanded in ***B***. Note that the oscillatory activity occurs at gamma frequency and shows a polarity reversal (indicated by vertical dashed lines) between P and R, as in adults (Buzsáki et al., 1983; Csicsvari et al., 2003). Also note the unit activity that is associated with the oscillation. ***C***, Power spectrum (in stratum pyramidale; 4096-point fast Fourier transform) of the oscillatory activity in ***B***. The activity shows a peak in the lower gamma-frequency range (~30 Hz). ***D***, Analyses of twitch-triggered gamma power in stratum pyramidale, stratum radiatum, and dentate gyrus for the same P7 subject. Vertical dotted lines at 0 ms indicate the time of twitch occurrence. Baseline (random-triggered) gamma power is shown in dark gray. Twitch-triggered gamma power is shown in light gray, and was considered significant when it exceeded the baseline mean + 3 SDs ($p < 0.002$; indicated by horizontal black line).

Data from a representative P7 subject are presented in Figure 4*A* to illustrate the coupling between hippocampal unit activity, the LFP, and AS-related twitching. A portion of the LFP record is expanded in Figure 4*B* to reveal the gamma rhythm, which exhibits peak power at ~30 Hz (Fig. 4*C*).

For each subject, we quantified the relationship between gamma activity and twitching. We considered gamma activity to be significantly associated with twitching when the average twitch-triggered gamma power was ≥3 SDs ($p < 0.002$) above the random-triggered (baseline) mean (see Materials and Methods). This analysis revealed that gamma power increased substantially immediately surrounding a twitch in all three hippocampal strata (Fig. 4*D*); twitch-related gamma power was stable between P1 and P7 and increased thereafter (Fig. 5). Finally, the proportion of subjects with significant coupling of gamma power and twitching peaked between P6 and P8 in all three hippocampal strata (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

We next determined whether unit activity exhibited phase locking to the gamma oscillations, as is observed in a minority of adult hippocampal neurons (Senior et al., 2008). Although brief, isolated instances of phase locking to the troughs of gamma oscillations were observed, such phase locking was never reliably expressed, and was not statistically significant even at the oldest ages examined here.

Bursts of unit and gamma activity during twitching give rise to the hippocampal theta rhythm

Theta activity first appeared at P8, and its amplitude increased with age (Fig. 5) (Leblanc and Bland, 1979). Data from a representative P8 subject are presented in Fig. 6*A* to illustrate the coupling between hippocampal unit activity, LFP activity, and AS-related twitching. A portion of the LFP record is expanded to reveal the theta rhythm (Fig. 6*B*), and a portion of that record is expanded further to reveal the imbedded gamma rhythm (Fig. 6*C*). With the onset of the theta rhythm, AS-related gamma activity began to be modulated at theta frequency, as it is in adults (Bragin et al., 1995b); gamma power increased at the age of theta onset as well (Fig. 5). Figure 6*D* further illustrates the relationship between the LFP and the theta and gamma rhythms during a period of twitching.

To quantitatively assess the relationship between AS and theta activity, we used the same procedure that was used to assess the relationship between AS and gamma activity. For the representative P8 subject analyzed in Figure 6*E*, theta power can be observed to increase substantially immediately surrounding a twitch in all three hippocampal strata (Fig. 6*E*). This tight coupling between theta power and twitching was present in all P8 subjects and persisted through P12 (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

We next determined whether unit activity exhibited phase locking to the theta oscillations, as it does in adults (O'Keefe and Dostrovsky, 1971; Ranck, 1973). At P8, 5 of the 16 recorded units (31.3%; 4 in CA1, 1 in DG) exhibited a significant phase preference, each firing during the trough of the theta oscillation (Fig. 7, left, note that because theta oscillations occur in brief bursts at P8, the trough at 0 ms is the most prominent aspect of the trough-triggered waveform average). These results suggest that immediately after its emergence at P8, the theta rhythm is capable of entraining unit activity in CA1 and DG.

At P9–P10, 4 of the 13 recorded units (30.8%; 3 in CA1, 1 in DG) exhibited a significant phase preference for the trough of the theta oscillation. By P11–P12, 8 of the 12 recorded units now exhibited a significant phase preference (66.7%; a significantly

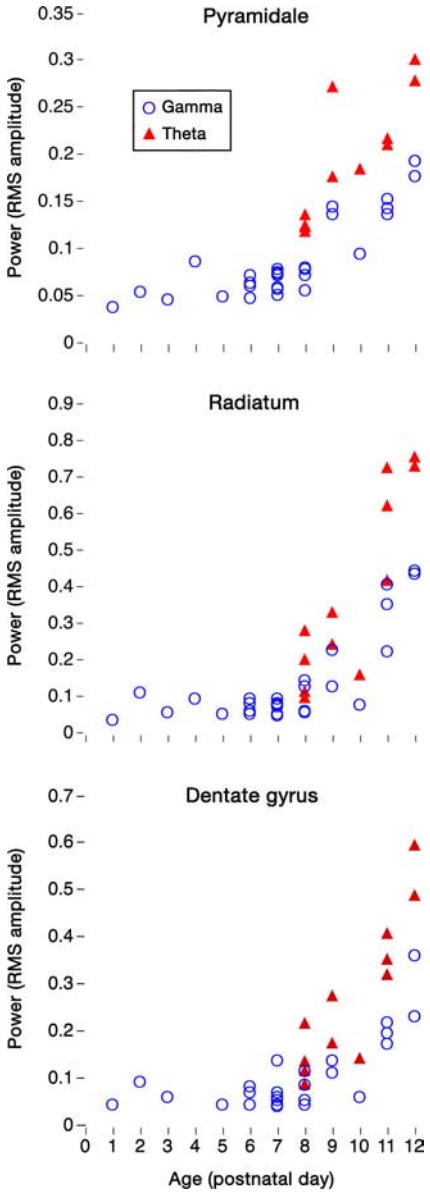

Figure 5. Developmental changes in AS-related gamma and theta power. Mean twitch-triggered gamma (20–100 Hz; open circles) and theta (4–14 Hz; filled triangles) power for each subject. Power values are derived from recorded activity in stratum pyramidale, stratum radiatum, and dentate gyrus. In each area, gamma power is consistently low until ~P8, the age at which theta first appears. Thereafter, gamma and theta power increase with age.

greater proportion than at P8 or P9–P10; $p < 0.025$, binomial test). Five of these units (four in CA1, one in DG) fired preferentially during the trough of the theta oscillation, whereas the remaining three units, all in DG, fired preferentially during the peak (Fig. 7, right panels).

Relationship between SPWs and QS-related gamma activity and population bursts

SPWs (also known as "giant depolarizing potentials" in neonates) are associated with both gamma tail activity (Mohns et al., 2007) and population bursts (Leinekugel et al., 2002) in the developing hippocampus. In the present study, we found that, as in adult rats, SPWs occurred most often during QS (Fig. 6*A*). SPW-associated gamma tails (first detected at P6) were therefore also a predominantly QS-related phenomenon, thus distinguishing them from the AS-related gamma activity (detected at all ages) that was associated with twitching. In addition, SPW-associated gamma tail activity tended to be higher in frequency (60–100 Hz) than twitch-related gamma activity (20–50 Hz). Finally, although we found that population bursts occurred most consistently during AS, they also did occur during QS-related SPWs (Leinekugel et al., 2002).

Discussion

In the present study, we found that the majority of neurons in the neonatal rat hippocampus fire during periods of AS-related myoclonic twitching, during which unit activity is significantly cross-correlated both within and across CA1 and DG. Moreover, a subset of these AS-active units fire preferentially within several hundred milliseconds after a twitch, thus suggesting that twitching during the early postnatal period provides sensory feedback that modulates activity not only within the spinal cord and neocortex (Petersson et al., 2003; Khazipov et al., 2004), but within the hippocampus as well.

Figure 8 illustrates the developmental progression of twitch-related hippocampal activity. Specifically, at P1, twitching is accompanied by bursts of unit activity; at P5, this unit activity is also accompanied by gamma-frequency oscillatory activity; at P8, theta activity is first detected and it occurs together with gamma and unit activity; finally, by P11, high-amplitude theta and gamma activity extend beyond periods of twitching, but continue to show amplitude and frequency increases during periods of twitching (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

Population bursts in the neonatal hippocampus are modulated by behavioral state

In the first study describing the firing patterns of neonatal CA1 neurons *in vivo* (Leinekugel et al., 2002), the association between neuronal activity and behavioral state was not quantitatively assessed. Here, by monitoring the tonic and phasic activity of multiple muscle groups, we find that the majority of hippocampal units are significantly more active during periods of AS-related myoclonic twitching. This preferential firing of neurons during AS may be a unique characteristic of infant hippocampal networks (for relevant work in adults, see Hirase et al., 2001b), perhaps reflecting a role for AS as a behavioral state that promotes the development of neural circuits (Roffwarg et al., 1966; Mirmiran, 1995; Blumberg and Lucas, 1996; Corner et al., 2002).

Our finding that CA1 and DG neurons *in vivo* are significantly cross-correlated is consistent with an *in vitro* developmental study by Crépel et al. (2007). The near-coincident firing of neuron pairs observed here appears to be a unique feature of neonatal hippocampal networks; during AS in adult rats, neurons do not cross-correlate at such a fine timescale (Kudrimoti et al., 1999; Hirase et al., 2001a). The augmented neuronal synchrony observed during the postnatal period has been attributed to the excitatory effects of $GABA_A$-receptor activation in neonates (Garaschuk et al., 1998), as well as electrical coupling by gap junctions (Crépel et al., 2007), which are more abundant during this period (Rozental et al., 2000; Vogt et al., 2005).

Importantly, the inclusion of behavioral measures in this study reveals that regularly recurring waves of hippocampal activity are modulated by the AS state *in vivo*. It is interesting to note that the correlated waves of activity in the neocortex of infant rodents (Garaschuk et al., 2000) are also expressed preferentially during "resting periods" (Adelsberger et al., 2005). However, the relationship, if any, between these neocortical activity patterns

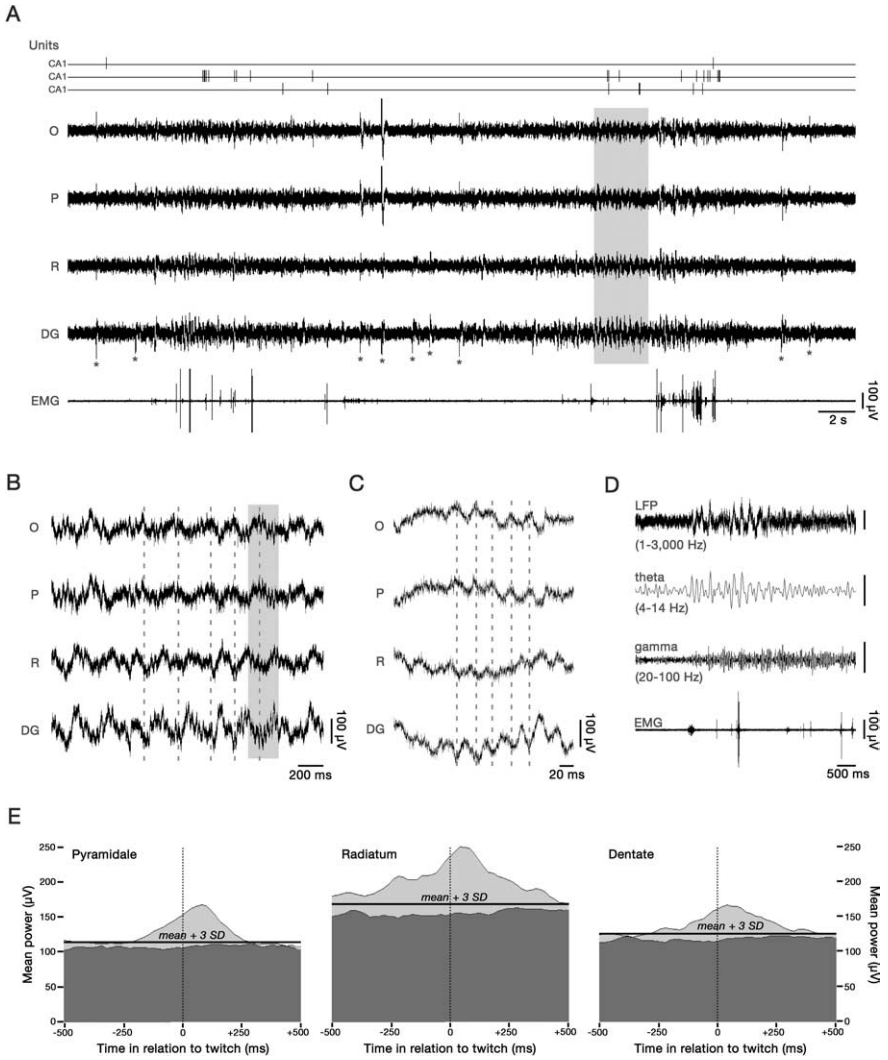

Figure 6. Emergence of the theta rhythm during AS-related twitches and unit bursts. ***A***, Data from a representative P8 rat, including unit activity from three simultaneously recorded neurons (top). LFP (1–3000 Hz bandpass) activity from strata oriens (O), pyramidale (P), radiatum (R), and DG is shown. Nuchal EMG activity is shown below. All bursts of activity visible in the EMG record signify twitching activity. Note that, as in Figure 4, LFP oscillatory activity and associated unit activity are associated with periods of AS-related twitches. Sharp waves (indicated by asterisks) are observed between bouts of twitching. The segment of data enclosed by the gray box is expanded in ***B***. ***B***, Unlike the P7 subject in Figure 4, the oscillatory activity now also includes theta-frequency activity. As indicated by the dashed lines, the theta activity exhibits a polarity shift between P and R, as in adults (Winson, 1974; Buzsáki, 2002). The area within the gray box is expanded in ***C***. ***C***, Gamma oscillations still reverse in polarity across P and R, but are now embedded within theta oscillations. Dashed lines again indicate the location of polarity reversal. ***D***, Another example from the same P8 subject, showing the coupling between theta- and gamma-frequency activity and AS-related twitches. All bursts of activity visible in the EMG record signify twitching activity. ***E***, Analyses of twitch-triggered theta power in stratum pyramidale, stratum radiatum, and dentate gyrus for the same P8 subject. Vertical dotted lines at 0 ms indicate the time of twitch occurrence. Baseline (random-triggered) theta power is shown in dark gray. Twitch-triggered theta power is shown in light gray, and was considered significant when it exceeded the baseline mean + 3 SDs ($p < 0.002$; indicated by horizontal black line). As with gamma activity (Fig. 4), theta activity is significantly associated with twitching in all three hippocampal areas.

and AS, or their relationship to the hippocampal patterns seen here, remains to be determined.

Emergence of the gamma rhythm during population bursts

The gamma rhythm emerged during the phasic population bursts that accompany AS-related twitching in neonates. This observation appears compatible with a previous *in vitro* study, which indicated that population bursts in the neonatal hippocampus give rise to transient field oscillations at gamma frequencies in CA3 (Palva et al., 2000). The present results indicate that the behavioral correlate of both population bursts and the consequent gamma activity is AS-related twitching. These results also are compatible with the finding of Lahtinen et al. (2001) that, at P6, gamma power in CA3 increases during AS.

In a previous study (Mohns et al., 2007), high-frequency oscillations (>60 Hz) in the hippocampus first appeared at P6 in the form of SPW-associated gamma tails (60–100 Hz). It was also noted that slower gamma activity (20–50 Hz) occurs as early as P2, although independently of SPWs (Karlsson et al., 2006). In the present study, we found that gamma tails occur primarily during QS, and that slower gamma activity occurs primarily during AS. Based on these findings, we hypothesize that the non-SPW-associated population bursts identified previously by Leinekugel et al. (2002) may be related to the AS-related population/gamma bursts described here. In contrast, the SPW-associated bursts described by Leinekugel et al. (2002) as a "tail" of unit activity may be the forerunner of the QS-related SPW gamma tail.

Emergence of the theta rhythm during the phasic activity of AS

The AS-related theta rhythm began to emerge at P8 in short bouts that were tightly coupled to twitches, population bursts, and gamma activity. By P11–P12, the appearance of theta was no longer exclusively associated with twitches. Our results extend those of Leblanc and Bland (1979) and Lahtinen et al. (2001). However, they are not consistent with the study of Karlsson and Blumberg (2003), which reported theta activity at earlier ages.

The data reported here suggest that theta is capable of entraining unit activity after its first emergence at P8, as it does in adult rats (Buzsáki, 2002). In adults, oscillation-related entrainment of neuronal activity appears to facilitate communication within and among brain areas (Fries, 2005; Axmacher et al., 2006; Womelsdorf et al., 2007). During early development, synchronizing activity in this way may help to establish the intrahippocampal and extrahippocampal connections necessary for the later emergence of more complex cognitive abilities that depend on efficient interregional and intraregional communication.

The inputs responsible for generating the hippocampal theta rhythm arise primarily from the medial septal region (MS) of the basal forebrain and the entorhinal cortex (EC). It appears that the MS provides CA1 pyramidal cells with rhythmic, interneuron-mediated somatic IPSPs (Freund and Antal, 1988; Cobb et al., 1995; Vertes and Kocsis, 1997), and the EC appears to simultaneously provide EPSPs to the distal dendrites of those same cells (Leung, 1984; Buzsáki, 2002). The combination of these two in-

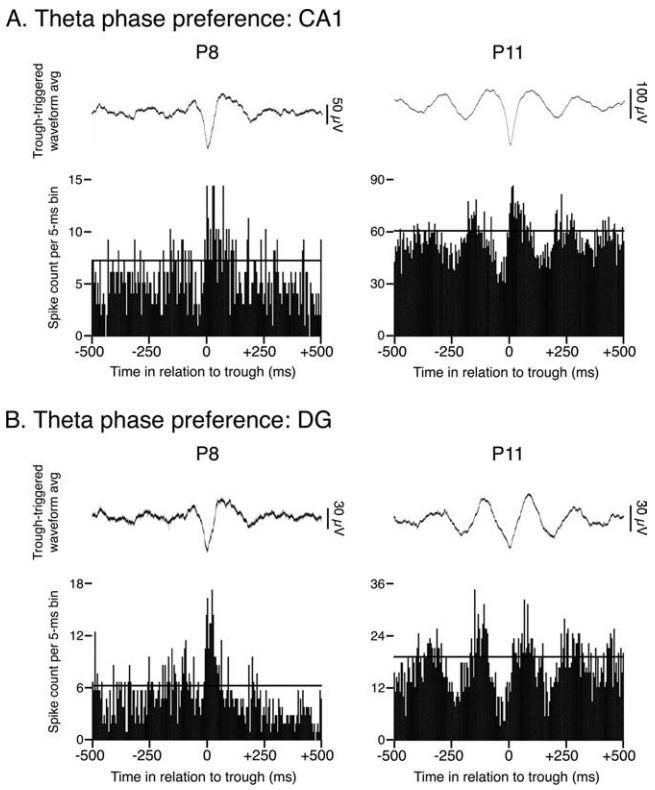

Figure 7. ***A***, ***B***, Examples of theta phase preference of single-unit activity at P8 and P11 in CA1 (***A***) and DG (***B***). Top traces, Trough-triggered waveform averages. Bottom traces, Trough-triggered spike count histograms. The horizontal line in each histogram indicates the baseline mean + 3 SDs ($p < 0.002$). Although units can fire preferentially during the theta trough at P8, this preferential firing has sharpened greatly by P11. Note that because theta oscillations occur in brief bursts at P8, the trough at 0 ms is the most prominent aspect of the trough-triggered waveform average. Also note that the P11 DG unit fires preferentially during the peak, rather than the trough of the theta oscillation; this was only observed in P11–P12 subjects.

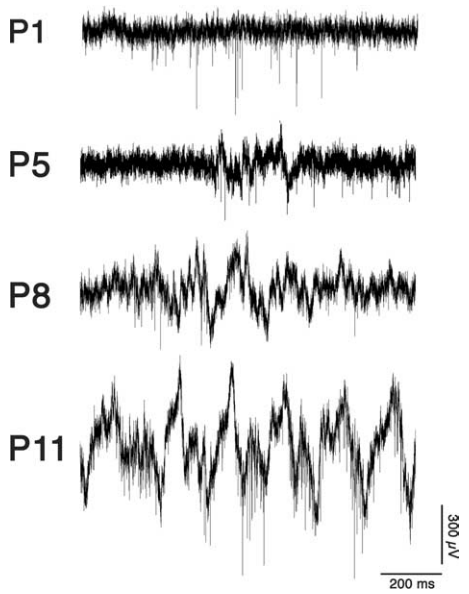

Figure 8. Developmental changes in hippocampal activity in relation to AS-related twitching. Representative examples of local field potentials (1–3000 Hz) from the pyramidal cell layer during periods of twitching in P1, P5, P8, and P11 rats. At P1, twitching is associated with unit activity without concurrent field oscillations. At P5, twitch-related unit activity occurs with gamma activity. At P8, gamma activity is still observed but is now embedded within the theta rhythm and both are tightly coupled with twitching. Finally, at P11, theta activity is more prominent during sleep and extends beyond the boundaries of twitching; unit activity is often phase locked with the theta rhythm.

puts may generate the phase and frequency characteristics that define the theta rhythm. However, the higher-frequency theta that accompanies active wakefulness and phasic AS (i.e., "type 1" theta) appears to be mediated predominantly by the EC inputs (Mitchell et al., 1982; Bland and Oddie, 2001), whereas the lower-frequency theta that accompanies awake immobility and tonic AS (i.e., "type 2" theta) appears to be mediated by the MS inputs (Kramis et al., 1975; Robinson et al., 1977). The initially tight coupling of the theta rhythm with twitches in neonates suggests that EC-mediated theta activity emerges several days before MS-mediated theta.

The appearance of the theta rhythm at P8 is most likely attributable to the rapid proliferation, beginning at ~P7, of perisomatic interneuronal synapses (Danglot et al., 2006). In addition, the cholinergic and GABAergic septohippocampal projections thought to be instrumental for type 2 theta generation are already present prenatally in rats, and increase in strength across the postnatal period (Bender et al., 1996). Furthermore, we have shown previously that basal forebrain neurons exhibit selective increases in activity during AS as early as P8 (Mohns et al., 2006). The developmental profile of the entorhinal efferents that mediate type 1 theta, however, has not yet been elucidated.

Functional implications

In adult rats, rhythmic membrane potential fluctuations associated with theta activity modulate the excitability of hippocampal neurons (Pavlides et al., 1988; Huerta and Lisman, 1995; Hölscher et al., 1997; Hyman et al., 2003; Vertes, 2005). The rhythmic potentiation and depotentiation of excitability may help to stabilize neuronal activity; indeed, theta appears to have antiseizure effects in adult rats (Miller et al., 1994; Colom et al., 2006). It is therefore interesting that the period of heightened seizure susceptibility in neonatal rats ends shortly after the emergence of the theta rhythm at P8 (Mohns et al., 2007).

It has been hypothesized that feedback from myoclonic twitches in infant rats contributes to the self-organization of somatotopy within the spinal cord (Petersson et al., 2003). In addition, Khazipov et al. (2004) demonstrated in P1–P6 rats that sensory feedback from AS-related twitches and locomotor activity produce distinct "spindle bursts" (SBs) in somatosensory cortex; they hypothesized that these twitch-SB events contribute to the development of somatotopy. Here, we have shown that a subset of hippocampal neurons is also activated after the production of myoclonic twitches. We hypothesize that twitch-related neocortical activity triggers this hippocampal activity, most likely via entorhinal inputs (Isomura et al., 2006).

Just as retinal waves provide structured sensory input that contributes to the development of the visual system (Katz and Shatz, 1996; Feller, 1999; O'Donovan, 1999), myoclonic twitching may provide structured tactile and proprioceptive input that contributes to the development of the somatomotor system. Moreover, the relationships between motor output and sensory return established within the infant neocortex and hippocampus may facilitate the formation of the egocentric maps, and ultimately the allocentric maps, that make spatial navigation possible (McNaughton et al., 1996, 2006). Thus, sensory feedback from myoclonic twitching may help to synchronize activity between the hippocampus and neocortex, thereby establishing the capacity for the integrative interactions that are central to the learning and memory functions of these forebrain structures (Lavenex

and Amaral, 2000; Sirota et al., 2003; Jones and Wilson, 2005; Siapas et al., 2005; Buzsáki, 2006; Ji and Wilson, 2007).

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