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Developmental emergence of transient and persistent hippocampal events and oscillations and their association with infant seizure susceptibility

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Abstract

During the second postnatal week in rats, the hippocampus exhibits a transient period of hyperexcitability. To systematically assess the relationship between the onset and end of this period and spontaneous hippocampal activity, we used silicon depth electrodes in unanaesthetized head-fixed rats from postnatal day (P)2 to P18. At all ages, hippocampal sharp waves (SPWs) were prominent in the EEG. Beginning at P6, however, marked changes in SPWs and associated oscillations were detected. SPW-related 'gamma tails' (60–100 Hz) and 'ripples' (140–200 Hz) were first observed at P6 and P7, respectively, and both oscillations persisted up to P18. Transiently, between P6 and P11, SPW duration decreased and the occurrence of SPW doublets increased. In addition, between P8 and P11, a subset of rats exhibited 'spontaneous potentiated SPWs' characterized by double polarity reversals, enhanced likelihood of gamma tails, and population spikes. Having identified a suite of transient hippocampal features consistent with a window of increased excitability, we next assessed whether electrographic seizure activity would be most easily induced during this period. To do this, kainic acid (KA; 200 ng/infusion) was infused into the hippocampus contralateral to the recording probe. KA did not induce seizure activity until P7 and reached peak effectiveness at P9. Thereafter, sensitivity to KA declined. All together, these findings provide *in vivo* neurophysiological support for the notion of a developmental window of heightened seizure susceptibility during the second postnatal week, and also suggest that spontaneous nonpathological hippocampal activity can be used to mark the onset and end of this period.

Introduction

Seizure susceptibility is greatest during the neonatal period in both humans and rats (Moshe, 1987; Hauser, 1995). In rats, peak susceptibility to hippocampal seizures occurs during the second postnatal week (Jensen *et al*., 1991; Swann, 1995, 2004; Schuchmann *et al*., 2006). Several mediating factors have been suggested to account for this increased susceptibility. Specifically, recurrent collateral branches of pyramidal cells abruptly reach levels greater than those seen in adulthood before gradually being refined (Gomez-Di Cesare *et al*., 1997), proteins that mediate electrical coupling between neurons are more abundant than in adults (Rozental *et al*., 2000; Vogt *et al*., 2005), and GABA-mediated inhibition has not yet emerged (Rivera *et al*., 1999). Finally, the transient up-regulation of glutamate receptors (Monyer *et al*., 1994; Ritter *et al*., 2002) and the concomitant increase in sensitivity to the epileptogenic effects of glutamate receptor agonists suggest a role for this neurotransmitter (Jensen, 1999, 2002; Sanchez & Jensen, 2001).

The excitability and synchrony of the adult hippocampus are reflected in the frequency and duration of hippocampal sharp waves (SPWs; Buzsaki, 1984, 1989). Accordingly, the transiently increased tendency of the neonatal hippocampus toward excitability and synchronization (Holmes & Ben-Ari, 1998) should be reflected in the expression of SPWs, and perhaps also their associated high-frequency oscillations. Two recent studies have examined the development of SPWs *in vivo*. In the first study, Leinekugel *et al*. (2002) studied rats at postnatal days (P)3–6, and reported that SPWs constituted the predominant form of hippocampal activity. In the second study, Buhl & Buzsaki (2005), testing P12–20 rats, reported that the SPW-associated 140–200 Hz 'ripple' (O'Keefe & Nadel, 1978; Suzuki & Smith, 1988; Buzsáki *et al*., 1992) does not emerge until P14. However, because these two studies did not examine subjects during the developmentally important second postnatal week, it remains unclear how age-dependent changes in hippocampal activity relate to the developmental onset and end of the period of hyperexcitability. A direct examination of this issue is fundamental to any hypothesis relating the developmental emergence of spontaneous high-frequency activity (> 40 Hz) to the pathological high-frequency oscillations (≥ 60 Hz) that characterize seizure activity (Khalilov *et al*., 2005; Le Van Quyen *et al*., 2006).

We therefore examined hippocampal activity *in vivo* in P2–18 rats. As expected, SPWs were prominent at all ages. Beginning at P6–7, however, marked changes in SPWs and their associated oscillations were detected, including the appearance of SPW-related 'gamma tails' (60–100 Hz) and ripples (140–200 Hz). In addition, several transient SPW-related features, consistent with hyperexcitability, were expressed between P7 and P11, suggesting that spontaneous activity in the hippocampus reveals its state of excitability and proneness to seizure. To test this notion *in vivo*, we administered successive

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infusions of kainic acid (KA; 200 ng/infusion) into the hippocampus of P5–16 rats contralateral to the recording probe. KA induced interictal activity and high-frequency oscillations most effectively at P7–10; it was ineffective at P6 and its efficacy declined after P10. All together, these findings provide a definitive developmental link between spontaneous hippocampal activity and heightened seizure susceptibility.

Materials and methods

All experiments were carried out 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. All efforts were made to minimize the number of animals used.

Subjects

Forty-nine P2–18 male Sprague–Dawley Norway rats (*Rattus norvegicus*) from 36 litters were used. Some of the data from five P2–4 subjects were reported in a previous study (Karlsson *et al*., 2006). 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 is day 0). Mothers and their litters were housed in standard laboratory cages (48 × 20 × 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 07.00 h, and all tests were conducted between 12.00 and 17.00 h.

Surgery

Under isoflurane anaesthesia, the subject's skull was exposed and then bleached, dried, and coated with Vetbond (3M, St Paul, MN, USA) to add strength. Next, to secure the subject's head during testing, a custom-built stainless steel apparatus (Karlsson *et al*., 2005), designed to attach to the earbar and nosebar holders of a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA), 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 artifacts. Electromyogram (EMG) electrodes (50 μm diameter; California Fine Wire, Grover Beach, CA, USA) were implanted into the subject's right nuchal muscle to identify behavioural state (Karlsson & Blumberg, 2002) as well as the left vastus lateralis muscle to help identify startles (see below). Finally, to inhibit movement and calm the subject, it was wrapped gently in gauze (Corner & Kwee, 1976; Karlsson *et al*., 2005). The subject then recovered for 1 h in a humidified incubator maintained at thermoneutrality.

Procedure

General recording procedure

After recovery from surgery, the subject was transferred to the recording apparatus. First, the subject was secured into the stereotaxic apparatus, after which the skull was leveled in the horizontal plane. After a 1-h acclimation period, the recording electrode was inserted ∼2 mm posterior to bregma and 1–2 mm lateral to midline. All waveforms were recorded during sleep, as indicated by nuchal muscle atonia (Karlsson *et al*., 2005); this was done to maintain consistency in terms of the behavioural conditions in which SPWs were recorded (it should be noted, however, that behavioural state did not appear to affect the characteristics of SPWs or SPW-associated events at any age). During all experiments, body and brain temperatures were maintained at ∼35 °C 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; Neuronxus Technologies, Ann Arbor, MI, USA) lowered into the dorsal CA1–dentate gyrus axis. An insulated silver wire (Medwire, Mount Vernon, NY, USA; 0.25 mm diameter), inserted into the cerebellum, served as both ground and reference electrode. Silicon probes were connected to a unity-gain headstage and digital amplifier (Tucker-Davis Technologies, Alachua, FL, USA) that amplified (×10 000) and filtered (1–5000 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, Carlsborg, WA, USA). Neural and EMG signals were sampled at 12.5 kHz using a digital interface (Cambridge Electronic Design, Cambridge, UK) and recorded synchronously to hard disk for off-line analysis using Spike2 software (Cambridge Electronic Design). Two to four consecutive 15-min recordings were made for each subject, and the recording for each subject that was determined to have the greatest number of SPWs was selected for analysis.

Kainic acid (KA) infusions

Thirteen of the P5–10 rats, as well as 14 additional rats (from 10 litters), received intrahippocampal infusions of KA to assess seizure susceptibility across development. After two consecutive 15-min baseline recording periods, a 30-gauge needle attached to a 10 μL Hamilton syringe was lowered slowly into the contralateral CA1 pyramidale–radiatum region at approximately the same coordinates as the recording electrode. Next, 1 μL of KA (200 ng/μL; dissolved in sterile saline; MP Biomedicals, Solon, OH, USA, and Tocris Cookson, Ellisville, MO, USA) was manually infused (1 μL/10 s). After each infusion, hippocampal activity was continuously recorded for 10 min. Each subject received a maximum of 10 infusions. Upon insertion of the syringe needle into the contralateral hippocampus, we detected no change in electroencephalogram (EEG) activity. It should also be noted that hippocampal seizures in these experiments did not typically entail marked motor activity; indeed, nuchal atonia was often observed.

Data analysis

Detection of SPWs

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 & Nadel, 1978; Buzsaki *et al*., 1983; Suzuki & Smith, 1987). Only events with peak negative amplitudes in stratum radiatum that were ≥3× the baseline activity were scored as SPWs (Karlsson & Blumberg, 2004). Although not quantified in the present study, SPWs were often accompanied by an increase in multiunit activity, as reported previously in neonates (Leinekugel *et al*., 2002). The rate of SPW occurrence was calculated for each subject as the total number of SPWs observed per min, and average SPW amplitude was calculated

as the mean baseline-to-peak negative amplitude of the waveform at 200 μm below stratum pyramidale (i.e. in stratum radiatum).

Current source density (CSD) analysis

Field patterns in all recorded channels were averaged using the identified negative peaks of SPWs. One-dimensional CSD analyses (Nicholson & Freeman, 1975; Mitzdorf, 1985) were performed and contour maps were generated using custom scripts written for Matlab 7.0 (MathWorks, Natick, MA, USA). The anatomical layers corresponding to the vertical scale of the CSD maps were reconstructed with the aid of the histologically identified electrode tracks and marking lesions.

Relationship between SPWs and startles

Because recent studies have indicated a temporal link between SPWs and behavioural 'startles' in the neonatal period (Karlsson & Blumberg, 2003; Karlsson *et al*., 2006), this temporal relationship was also assessed in the present study. Startles are sudden, spontaneous and simultaneous contractions of multiple muscle groups throughout the body and are particularly prominent in early infancy in rats (Gramsbergen *et al*., 1970). We have found previously that EMG activity recorded simultaneously from multiple muscle groups can reliably be used to reveal these events (Karlsson *et al*., 2006). For the present study in which EMG activity was recorded from each subject's right nuchal and left vastus lateralis muscles, a startle was defined as the co-occurrence of EMG spikes (≥ 3× baseline) in both muscle groups with an interspike latency ≤ 70 ms. A startle was considered to co-occur with a SPW when it preceded the SPW by ≤ 225 ms (Karlsson *et al*., 2006).

SPW doublets and SPW duration

A SPW doublet was defined as two complete SPWs occurring within a 400-ms time window (Bragin *et al*., 1995). SPW duration (i.e. the width of the negative deflection from baseline) was determined for the recording site 200 μm below stratum pyramidale, and the average for each subject was calculated. The total number of doublets per min and the average SPW duration were calculated for each subject.

Identification of SPW-related high-frequency oscillations

Observed SPW-related waveforms in the local field potential (LFP) recordings included 140–200 Hz ripples (Buzsáki *et al*., 1992; Ylinen *et al*., 1995; Buhl & Buzsaki, 2005) and 60–100 Hz gamma 'tails' that closely followed SPW negative peaks (Suzuki & Smith, 1988; Bragin *et al*., 1995; Traub *et al*., 1996). Analyses thus focused on activity within the ripple- and 'fast gamma'-frequency ranges (i.e. using 140–200 and 60–100 Hz bandpass filters, respectively). Ripples were considered SPW-related if they occurred within ± 50 ms of the SPW negative peak; gamma activity was considered a gamma tail if it occurred within 100 ms of the SPW negative peak. In order for a filtered waveform to be selected for analysis, it was required to (i) exhibit an amplitude ≥ 2× baseline activity, (ii) exhibit three complete, continuous cycles, each with clear polarity reversals (180 ± 30°) across stratum pyramidale, and (iii) propagate at least 300 μm above (i.e. into stratum oriens) and below (i.e. into stratum radiatum) stratum pyramidale. Conditional probabilities of ripples and gamma tails given the occurrence of a SPW (i.e. p(x|SPW)) were calculated for each subject.

Although not specifically addressed in the present study, theta activity (4–12 Hz) and dentate spikes were also observed in the dorsal hippocampus. Theta activity first emerged at P8, consistent with a previous report (Leblanc & Bland, 1979), and was characterized by a polarity shift between stratum oriens and the hippocampal fissure (Winson, 1974; Buzsáki, 2002). Dentate spikes were first observed at P7, consistent with a previous report (Karlsson & Blumberg, 2004), and exhibited polarity reversals near the molecular layer of the dentate gyrus (Bragin *et al*., 1995).

Identification of seizure activity

The onset of seizure activity was defined as the onset of the first interictal episode, identified by the presence of interictal spikes (IISs). IISs were easily identified on the basis of their shape and laminar profile (see Results for details). Although single IISs can occur in isolation, in our experiments their initial appearance was always characterized by a series of 10 or more spikes occurring at a rate > 1 Hz, thus constituting what we defined as an interictal episode. In older subjects, these interictal episodes often transition directly into tonic and/or clonic discharges typical of adult seizure activity (Khalilov *et al*., 1999; Khazipov *et al*., 2004). Latency to exhibit seizure activity was thus quantified as the number of KA infusions administered before the appearance of IISs. To test the hypothesis that P7–10 marks a period of heightened sensitivity to KA infusion, subjects were categorized as KA-sensitive (defined as exhibiting seizure activity after four or fewer infusions) or KA-insensitive (defined as not exhibiting seizure after 10 infusions). Fisher's exact probability test was then used to test the hypothesis that P7–10 rats were more likely to exhibit KA-induced seizure activity than younger or older subjects.

CSD analyses of IISs were performed as with SPWs, triggered by peaks in stratum radiatum. Associated pathological high-frequency oscillations were first identified by eye in the LFP (1–5000 Hz) traces and then quantified after bandpass filtering (60–200 Hz).

Histology

Following each experiment, 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 phosphate-buffered saline, 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 (as well as needle tracks, where applicable) and marking lesions for each subject, and to reconstruct the anatomical locations of the recording sites.

Results

Characterizing hippocampal SPWs between P2 and P18

SPWs exhibited polarity reversals across stratum pyramidale, as documented by others (Ylinen *et al*., 1995; Leinekugel *et al*., 2002). Figure 1A illustrates these polarity reversals in representative P2, P10 and P18 subjects. CSD analyses revealed that, at all ages examined, neonatal SPWs exhibited an inward current (sink) in stratum radiatum and an outward current (source) in stratum pyramidale, similar to those that characterize SPWs in adults (Ylinen *et al*., 1995). Rate and average negative amplitude of SPWs are presented in Fig. 1B and C, respectively.

It was recently reported in P1–4 rats that SPWs are reliably preceded by behavioural 'startles', defined as phasic, simultaneous

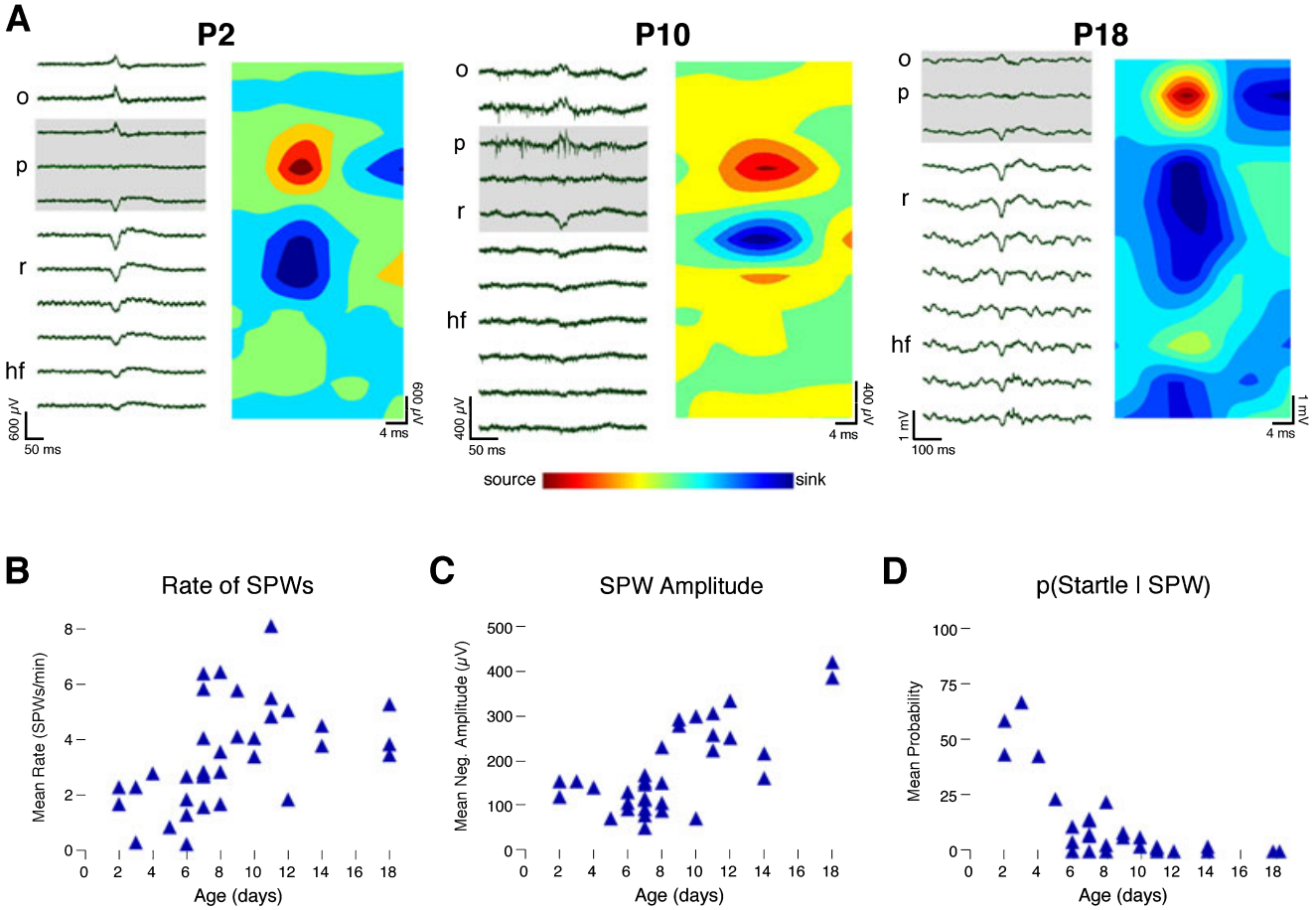

FIG. 1. SPW profiles across the early postnatal period. (A) For representative subjects at P2, P10 and P18, depth profiles of single SPWs (left panels) and CSD plots (right panels) are presented. Signals were recorded using silicon probes (1–5000 Hz bandpass). CSD plots were constructed by averaging across all SPWs (n = 23–66 per subject). Outward currents (sources) and inward currents (sinks) are indicated by warm and cool colours, respectively. Note polarity reversals across stratum pyramidale (p) in wide-band traces (grey boxes) with corresponding sources in the CSD plots at each age; also note sinks in stratum radiatum (r) at all ages. (B) Average number of SPWs per min for each subject. (C) Average negative amplitude of SPWs, measured in stratum radiatum, for each subject. (D) For each subject, the conditional probability that a SPW was accompanied by a startle. o, stratum oriens; p, stratum pyramidale; r, stratum radiatum; hf, hippocampal fissure.

contractions of multiple skeletal muscles throughout the body (Karlsson *et al.*, 2006). Here we found that this relationship diminished rapidly with age such that, by P9, startles persisted but now rarely co-occurred with SPWs (Fig. 1D).

A representative SPW doublet from a P8 subject is shown in Fig. 2A. Doublets were rarely observed before P6, increased abruptly between P7 and P10, and then declined after P11. Figure 2B illustrates the transient decrease in SPW duration that occurred during the same developmental period.

Developmental emergence of high-frequency oscillations in association with SPWs

Figure 3A presents recordings from representative subjects at P2, P8 and P18. For each subject, LFP traces from stratum radiatum (top trace) are presented to indicate the occurrence of SPWs, as well as filtered data from stratum pyramidale (bottom trace; 60–200 Hz bandpass) to reveal associated bursts of high-frequency activity within the ripple- and gamma-frequency ranges. Although the P2 subject exhibited SPWs (indicated by asterisks in the LFP trace), the lower trace shows that there was no associated high-frequency activity. By P8, SPWs were intermittently accompanied by low-amplitude high-frequency oscillations; note also the increased amount of non-SPW LFP activity. By P18, SPWs were more consistently associated with bursts of high-frequency activity, and high-frequency oscillations often occurred independently of SPWs. Moreover, at P18 the amplitude of both SPWs and high-frequency oscillations was greater, as was the baseline level of activity.

The high-frequency activity accompanying neonatal SPWs could be separated into two distinct waveforms: ripples and gamma tails. Ripple-frequency activity (i.e. 140–200 Hz) during SPWs was first observed at P7. The left panel of Fig. 3B presents a laminar profile of a prominent P7 ripple. The grey box in the LFP traces indicates the location of the ripple, which is shown to the right in expanded and filtered (140–200 Hz) form. Note the polarity reversals in the filtered traces. The middle panel presents a typical P18 ripple; note the increased amplitude and number of constituent oscillations. The conditional probability of observing a ripple given the occurrence of a SPW for each subject is presented in the right panel of Fig. 3B. This probability was initially low but increased steadily until P14 when it reached adult levels (Bragin *et al.*, 1999a).

Gamma tail activity (i.e. 60–100 Hz) was first observed at P6. At earlier ages, the highest frequency oscillation present in the hippocampal EEG was ‘slow gamma’ activity (i.e. 20–30 Hz), which was never observed in association with SPWs (Karlsson *et al.*, 2006). The left panel in Fig. 3C presents a laminar profile of a representative

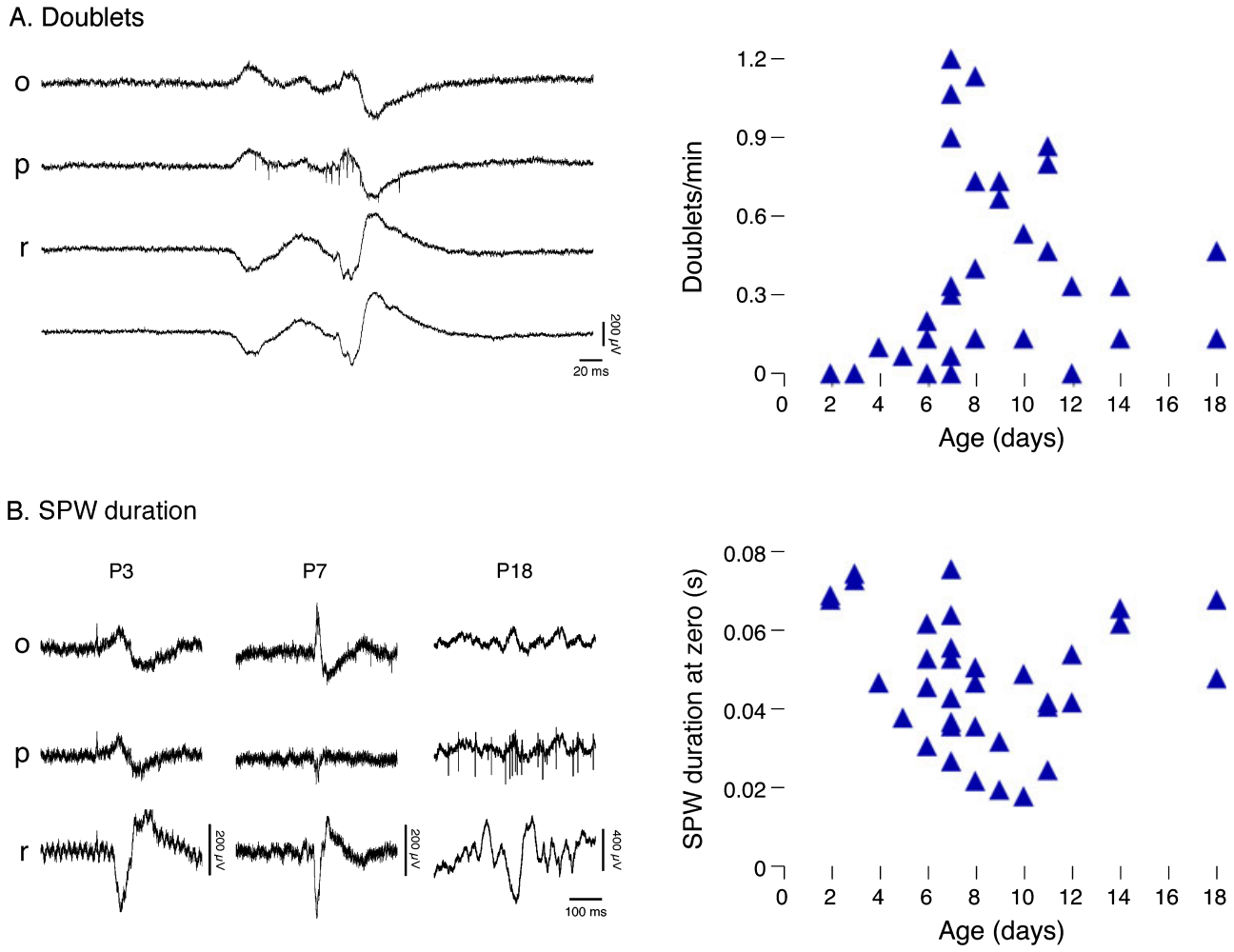

FIG. 2. Developmental changes in the occurrence of SPW doublets and SPW duration. (A, left panel) A representative SPW doublet in a P8 subject. (Right panel) The number of SPW doublets per min for each subject. (B, left panel) Representative examples of SPW duration at P3, P7 and P18. (Right panel) Average SPW duration for each subject. Abbreviations as in Fig. 1.

gamma tail in a P8 subject. The grey box in the LFP traces indicates the location of the gamma tail, which is shown to the right in expanded and filtered (60–100 Hz) form. Note the polarity reversal of the gamma tail below stratum pyramidale, just as is the case for ripples.

The middle panel of Fig. 3C illustrates gamma tail activity at P18. Similar to the developmental trend seen for ripples, the gamma tail at P18 has greater amplitude as well as a greater number of constituent oscillations. The right panel presents the conditional probability of observing a gamma tail given the occurrence of a SPW. This probability was low at P6 and remained low across development, with the exception of the five subjects between P8 and P11 highlighted in the figure. Upon closer inspection, these five subjects exhibited a number of additional distinguishing characteristics, detailed in the next section.

Spontaneous potentiated SPWs

Closer examination of the five subjects (all from different litters) highlighted in Fig. 3C revealed several distinguishing features. First, all SPWs in these subjects exhibited double polarity reversals: In addition to the typical polarity reversal observed at the pyramidale–radiatum border, there was a second polarity reversal above the hippocampal fissure, at the level of stratum lacunosum–moleculare (Fig. 4A, left panel). Previous studies in adult rats have documented a shift toward positive polarity at this level (Buzsaki *et al*., 1983; Buzsaki, 1986); however, the very prominent reversal (≥ 3× baseline) seen here appeared more similar to that observed in commisurally evoked SPWs in adults (Ylinen *et al*., 1995). CSD analyses revealed typical source–sink pairs at strata pyramidale and radiatum, but additionally revealed mirror sink–source pairs at the hippocampal fissure, corresponding to the second SPW polarity reversal at this level (Fig. 4A, middle panel). SPW double polarity reversals were observed in every SPW for these five subjects, whereas they were not observed in any other subjects (Fig. 4A, right panel).

A second distinguishing factor, as previously indicated in Fig. 3C, was the unusually high proportion of SPWs with gamma tails. An average of 89% of SPWs in these five subjects exhibited gamma tails (Fig. 4B, right panel). These gamma tails also exhibited double polarity reversals: one below stratum pyramidale of CA1 and a second between strata moleculare and granulosum of the dentate gyrus (Buzsaki *et al*., 1983; Csicsvari *et al*., 2003). Thus, while gamma tails and SPWs both reversed in polarity across stratum pyramidale, the second polarity reversal of gamma tails was ∼100 μm lower than the second polarity reversal of SPWs. All gamma tails in these five subjects exhibited double polarity reversals (≥ 2× baseline), whereas

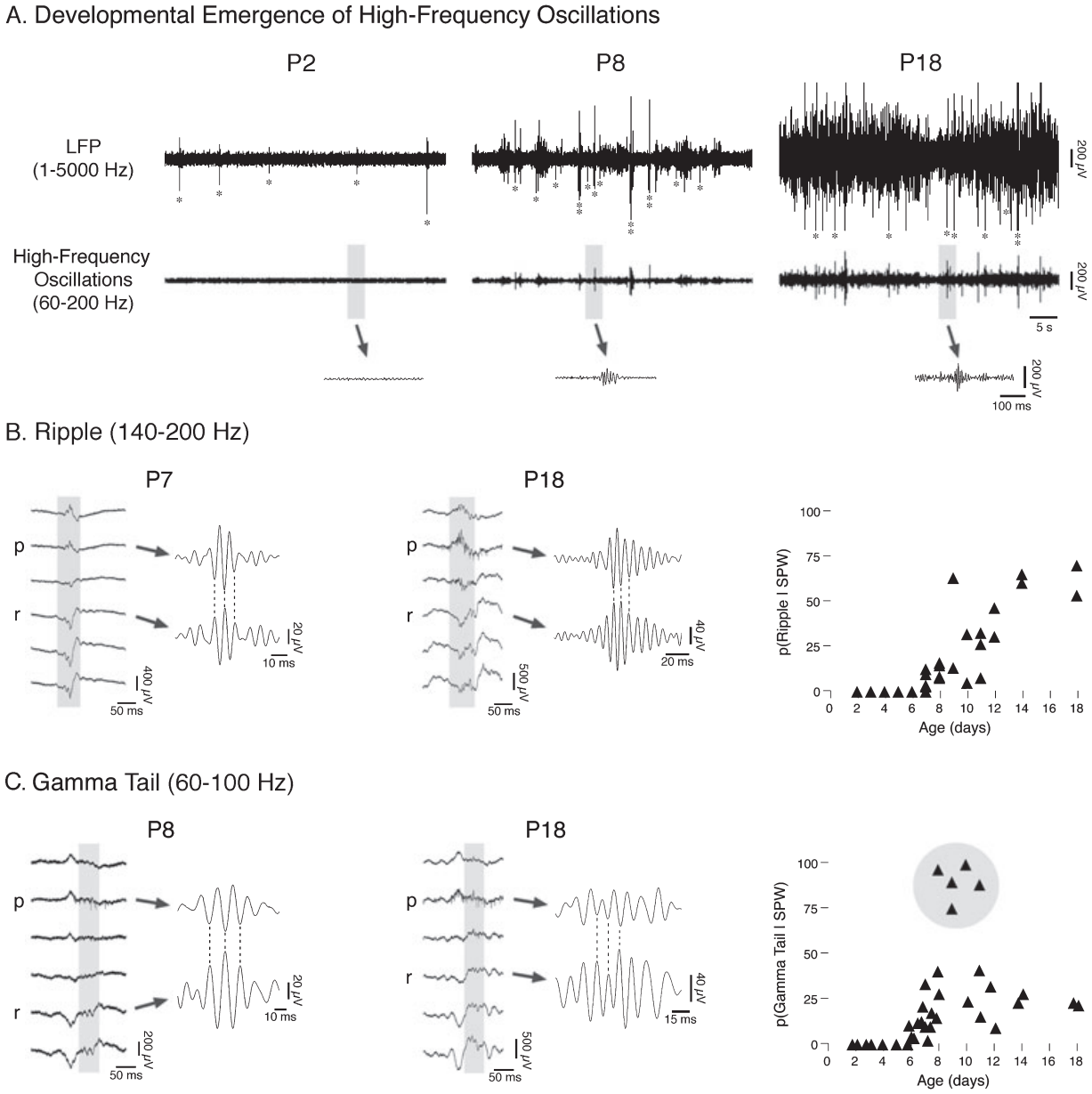

FIG. 3. Developmental emergence of SPW-related high-frequency oscillations. (A, Top trace) LFPs (1–5000 Hz bandpass) recorded from stratum radiatum in representative P2, P8 and P18 subjects. SPWs are indicated by asterisks and SPW doublets by vertical double asterisks. (Bottom trace) Filtered (60–200 Hz bandpass) traces from stratum pyramidale to reveal developmental emergence of high-frequency oscillations. Gray boxes indicate regions presented in greater detail below. (B) Representative SPW-associated ripples in P7 and P18 subjects. At each age, LFPs are shown; grey boxes indicate regions presented at right after filtering (140–200 Hz bandpass). Plot at far right panel presents, for each subject, the average probability of detecting a ripple given a SPW. (C) Representative SPW-associated gamma tails in P8 and P18 subjects. At each age, LFPs are shown; grey boxes indicate regions presented at right after filtering (60–100 Hz bandpass). Plot at far right presents, for each subject, the average probability of detecting a gamma tail given a SPW. Five P8–11 subjects are highlighted based on their exhibiting pronounced increases in the probability of SPW-related gamma tails (see Fig. 4 for details). Abbreviations as in Fig. 1.

gamma-tail double-polarity reversals were never observed in the other subjects.

Finally, SPWs in these five subjects occasionally contained a population spike during their initial deflection from baseline (Fig. 4C, left and middle panels). As described in adults, population spikes are very brief (< 5 ms), sharp, field potential spikes that result from the near-simultaneous discharge of a homogeneous cell population (Andersen *et al*., 1971). Here, such events were scored as population spikes when they exhibited negative amplitudes ≥3× baseline in stratum radiatum (200 μm below stratum pyramidale). Population spikes always reversed in polarity near stratum pyramidale, suggesting that they were generated in the perisomatic region. The percentage of SPWs with population spikes was low in these five subjects (mean ± SEM, 20.4 ± 3.5%), but their appearance is noteworthy because population spikes were never observed in other subjects (Fig. 4C, right panel).

Because double polarity reversals, gamma tails and population spikes are properties often associated with evoked (and, hence, potentiated) SPW responses in CA1 (Leung *et al*., 1995; Ylinen *et al*., 1995; Traub *et al*., 1996; Penttonen *et al*., 1998; Kloosterman *et al*., 2004), we hereafter refer to SPWs possessing these characteristics as 'spontaneous potentiated SPWs' (spSPWs).

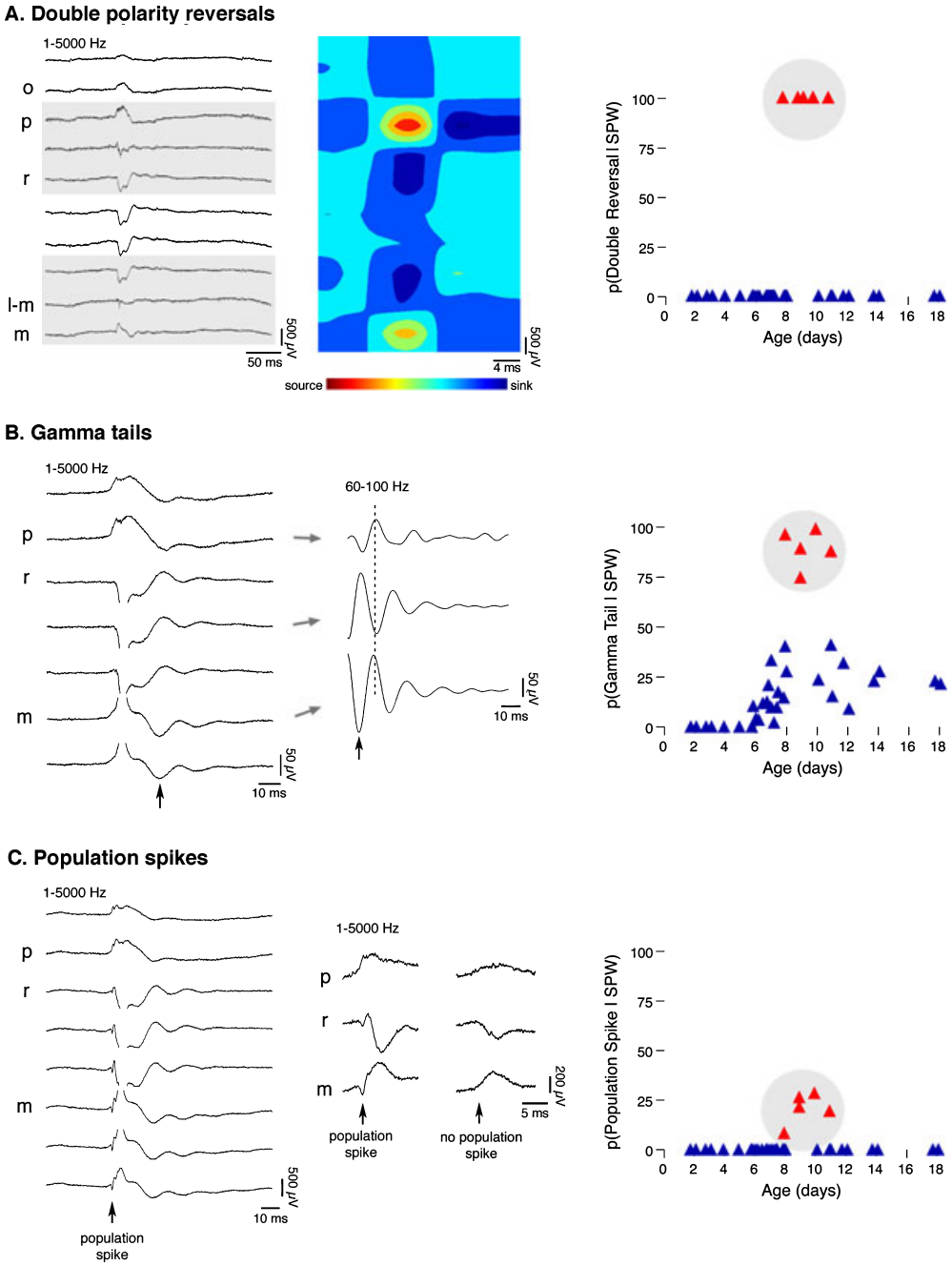

FIG. 4. Spontaneous potentiated SPWs. (A, left panel) Local field potentials (LFPs; 1–5000 Hz bandpass) indicating a spSPW in a P9 subject. The grey boxes highlight two polarity reversals: one across stratum pyramidale (p) and the other across stratum lacunosum–moleculare (l-m). (Middle panel) CSD plot revealing two pairs of sources and sinks corresponding to the two polarity reversals in the LFPs. (Right panel) For each subject, the average probability of observing a double-polarity reversal given a SPW. (B, left panel) LFPs for a P11 subject, illustrating a spSPW with associated gamma tail. To the right is a filtered (60–100 Hz bandpass) and expanded view of the same gamma tail. spSPW-related gamma tails, like spSPWs, always exhibited polarity reversals in two locations (highlighted by dashed line). Such gamma-tail double-polarity reversals were not observed in any other subjects. (Right panel) For each subject, the average probability of observing a gamma tail given a SPW. (C, left panel) LFPs for a P10 rat illustrating a spSPW with associated population spike (arrow). To the right is an expanded view of this population spike as well as a spSPW from the same subject that did not exhibit a population spike. The population spike appears to originate in stratum pyramidale (p), as indicated by its polarity reversal across this layer. (Right panel) For each subject, the average probability of observing a population spike given a SPW. m, molecular layer of dentate gyrus; all other abbreviations as in Fig. 1.

Age-dependent effects of repeated intrahippocampal kainic acid infusions in vivo

We hypothesized that the developmental onset and refinement of SPW-related patterns indicative of increased excitability and synchrony would be mirrored by changes in seizure susceptibility. Therefore, in a subset of the subjects just described, and in 14 additional subjects, we repeatedly infused KA into the hippocampus contralateral to the recording probe while continuously monitoring electrographic activity (Fig. 5A).

During the preinfusion period, all subjects exhibited typical EEG activity with normal SPWs characterized by polarity reversals across stratum pyramidale. In contrast, after KA infusion the onset of seizure

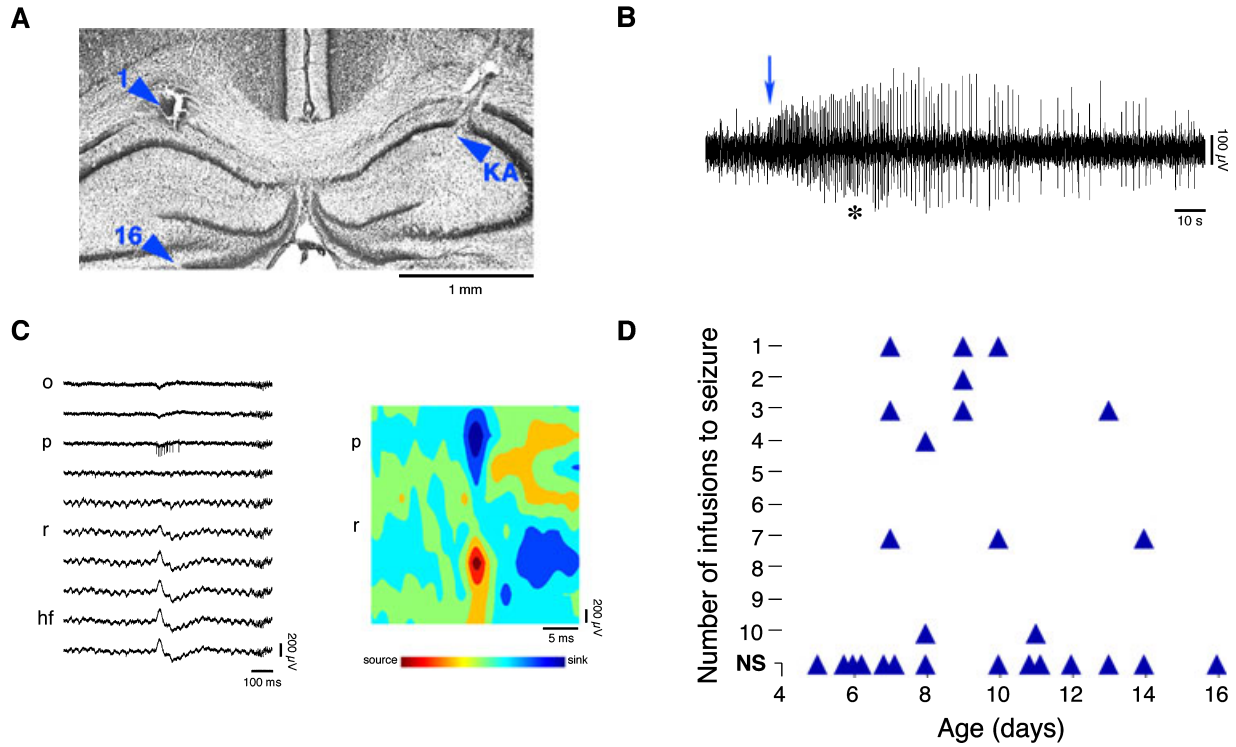

FIG. 5. Hippocampal activity following contralateral kainic acid infusion. (A) Histological section from a P8 subject, illustrating the design of the experiment. In the left hippocampus, a marking lesion indicates the location of the most dorsal recording site (site 1). The most ventral recording site is also indicated (site 16). In the right hippocampus, the location of the KA infusion is indicated by the needle track penetrating CA1. (B) For the subject in A, this recording illustrates the onset (indicated by arrow) of seizure activity following KA infusion. Asterisk indicates location of the IIS presented in C. (C, left panel) Representative depth profile (wide-band: 1–5000 Hz) of a type 2 IIS, and associated neuronal discharge in stratum pyramidale. Note the reversed polarity of this spike when compared to the SPWs in Fig. 1A. (Right panel) CSD plot derived by averaging across all IISs ($n = 19$) comprising the interictal episode from which the IIS in the left panel was selected. Note the reversed orientation of the source and sink in comparison to the CSD profiles for the SPWs depicted in Fig. 1A. (D) For each subject, the number of KA infusions required to evoke seizure activity. NS, no seizure activity detected after a maximum of 10 infusions. None of the P8–10 subjects infused with KA exhibited spSPWs. Abbreviations as in Fig. 1.

activity was characterized by the appearance of regularly recurring IISs (Khalilov *et al.*, 1999). Because the transition to seizure-like episodes was always characterized by rapid IISs, rather than an increase in the appearance of any particular oscillation, the transition was more akin to 'hypersynchronous' seizure onset than to 'low-voltage fast' seizure onset (Bragin *et al.*, 1999b, 2005; Velasco *et al.*, 2000). The mean peak frequency of these IISs was 3.82 ± 0.48 Hz, similar to the frequency reported previously in P8–11 rats during hyperthermia-induced seizures (Schuchmann *et al.*, 2006). It is noteworthy that none of the subjects exhibited prominent seizure-related motor activity, similar to adult rats exhibiting hypersynchronous seizure activity (Bragin *et al.*, 1999b).

In 11 of 18 subjects displaying seizure activity, IISs exhibited polarity reversals across stratum pyramidale opposite to those of SPWs, consistent with type 2 IISs (Fig. 5C; Wadman *et al.*, 1983; Buzsaki *et al.*, 1989, 1991; Leung, 1990). Their CSD plots indicated a source–sink pattern opposite to that exhibited by SPWs (Fig. 5C; see Fig. 1A for comparison). In contrast, the remaining seven subjects displayed type 1 IISs, which have a shape and laminar profile that is very similar to that of SPWs but with duration <40 ms (Wadman *et al.*, 1983; Buzsaki *et al.*, 1989, 1991; Leung, 1990). The duration of type 1 IISs was typically 20–30 ms.

Using the criteria just described, we determined the number of infusions required to evoke seizure activity in each subject. The results of this analysis, presented in Fig. 5D, indicate a developmental profile that mirrors those presented earlier for SPW doublets, SPW duration and spSPWs. Specifically, 200 ng/μL KA did not evoke seizure activity before P7, even after 10 infusions; indeed, before P7, no IISs or other EEG abnormalities were observed at any time during or after KA infusions. Seizure activity was first detected at P7. Sensitivity to KA infusions increased dramatically between P7 and P10 and declined thereafter such that, in most subjects, even 10 KA infusions were no longer sufficient to evoke seizure activity. This increased susceptibility of P7–10 rats in relation to younger and older subjects was statistically significant (Fisher's exact test, two-tail, $P < 0.05$).

The characteristics of seizure activity also changed with age. At P7, three of six subjects exhibited seizure activity. In these three subjects, the activity consisted of type 2 IISs with superimposed high-frequency oscillations similar to those reported in adult rats after KA infusion (Bragin *et al.*, 1999a; Bragin *et al.*, 2004). After P7, these high-frequency oscillations were observed in every subject that exhibited IISs. In one of the three P7 subjects displaying seizure activity, rapid IISs were not followed by tonic or clonic components, as has been demonstrated in P2–3 rats *in vitro* (Khalilov *et al.*, 1999). In the two other P7 subjects, the initial increase in IISs led to clonic-like multispike bursts (Bragin *et al.*, 1999b).

At P8, two of three subjects exhibited seizure activity. One subject displayed only rapid type 2 IISs without tonic or clonic components (Fig. 6B). The other subject displayed a transition from type 2 IISs to a larger waveform characterized by 100–200 Hz bursts that were followed by a tail of activity in the slow gamma- (20–50 Hz) and beta-frequency (10–20 Hz) ranges (Fig. 6C).

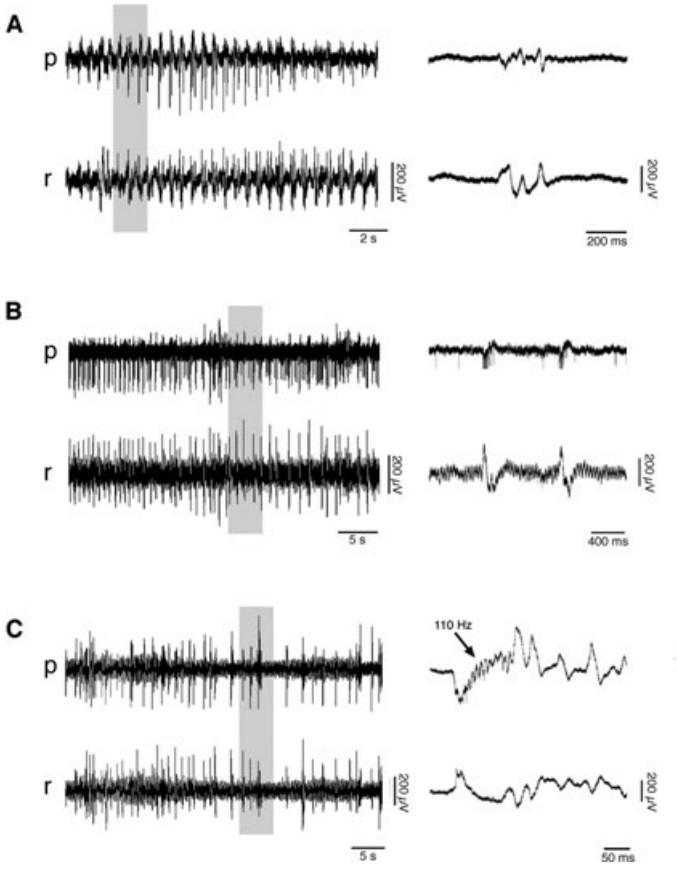

FIG. 6. Representative hippocampal seizure activity evoked by kainic acid infusion. Depicted are three general types of seizure activity displayed by subjects after kainic acid infusions into the contralateral hippocampus. In each case, local field potentials (1–5000 Hz bandpass) from strata pyramidale (p) and radiatum (r) are presented on the left, and grey boxes highlight portions magnified on the right. (A) An episode in a P7 subject characterized by single type 2 IISs, transitioning into clonic-like bursts. (B) An episode in a P8 subject characterized only by the appearance of rapid type 2 IISs. This subject was unique in showing multiunit activity in stratum pyramidale synchronized with the IISs. Also note the continuous 30-Hz rhythm in stratum radiatum; beginning at P8, all subjects displayed this potentiated gamma activity after 1–2 infusions of kainic acid, regardless of whether they exhibited seizure activity. (C) This P8 subject exhibited a progression from single type 2 IISs with no high-frequency oscillations to single IISs with high-frequency oscillations, to IISs with high-frequency oscillations that were followed by a gamma-beta tail.

The seizure activity in six of seven subjects at P9–10 consisted of type 1 IISs. Three of these subjects exhibited only an increase in the frequency of IISs, one subject exhibited rapid IISs which transitioned into clonic-like multispike bursts, and the remaining three subjects displayed highly synchronized tonic–clonic seizure activity, characterized by an initial interictal episode followed by alternating periods of 10–30 Hz 'tonic' oscillations and clonic discharges, finally terminated by high-amplitude clonic bursts (Khalilov *et al*., 1999). After P10, when seizure activity occurred it consisted of either type 1 or type 2 IISs, typically transitioning into tonic–clonic seizure activity.

Discussion

As summarized in Fig. 7, this study documents the developmental onset of both persistent and transient physiological patterns in the CA1 field of the hippocampus. The persistent patterns, which include the onset of SPW-related gamma tails and ripples at P6–7, coincide with the developmental onset of KA-induced seizure activity. The transient patterns, which include decreases in SPW duration, increased occurrence of SPW doublets and the expression of spSPWs, mirror the period of increased seizure susceptibility, framing both its onset and end. These results indicate that spontaneous hippocampal activity can be used to assess the state of excitability of the infant hippocampus.

SPW-related ripples and gamma tails

We detected ripples ~7 days earlier than was reported recently by Buhl & Buzsaki (2005). This inconsistency may be due to methodological differences between our two studies. In P12 and older rats, Buhl & Buzsaki (2005) used power-spectral analyses to determine the age of ripple onset. This method, however, may not be adequate for detecting ripples at earlier ages when, as shown here, ripples occur with SPWs only sporadically and are expressed at low amplitude. Regardless, the present results resolve an outstanding paradox by bringing into alignment the onset of both pathological and physiological high-frequency activity (60–200 Hz) at ~P7 (Le Van Quyen *et al*., 2006).

Although non-SPW-related gamma-frequency rhythms have been described in head-fixed and freely moving rats as early as P2–5 (Lahtinen *et al*., 2001; Karlsson *et al*., 2006), these bouts of gamma activity are of lower frequency (i.e. 20–60 Hz) than the SPW-related high-frequency (60–100 Hz) gamma tails that, as shown here, first appear at P6. The emergence of gamma tails at P6 may result from developmental changes in the glutamatergic network (Le Van Quyen *et al*., 2006). Indeed, because high-frequency gamma oscillations are dependent, in part, upon AMPA receptor-mediated excitation (Fisahn *et al*., 1998; Mann *et al*., 2005), it is noteworthy that the emergence of these oscillations at P6 is coincident with the associated up-regulation of AMPA receptors at this age (Sanchez & Jensen, 2001).

SPW doublets and duration

Hippocampal SPWs occur during periods of reduced cortical and subcortical inhibition (Chrobak & Buzsaki, 1994). It is for this reason that lesions of the entorhinal cortex enhance the rate of occurrence of SPWs and SPW doublets (Bragin *et al*., 1995), reflecting a state of increased excitability. In particular, SPW doublets are occasionally observed when perforant path stimulation is timed to the occurrence of spontaneous SPWs (Buzsaki, 1989). Doublets are also observed in rats with subcortical denervation of the hippocampus (Buzsaki *et al*., 1991).

SPW duration in CA1 is an indicator of the degree of synchrony among CA3 pyramidal neurons, with shorter durations reflecting greater synchrony (Buzsaki, 1984, 1989). Our finding that SPW duration was shortest during the second postnatal week is therefore consistent with the finding of Gomez-Di Cesare *et al*. (1997) that CA3 pyramidal cell collateralization is greatest during this period. In addition, because seizure itself is a product of neuronal network hypersynchrony, it is noteworthy that the period of shortest SPW duration was also coincident with the period of greatest seizure susceptibility reported here.

spSPWs

A subset of subjects between P8 and P11 exhibited spSPWs, characterized by double polarity reversals and, less often, population

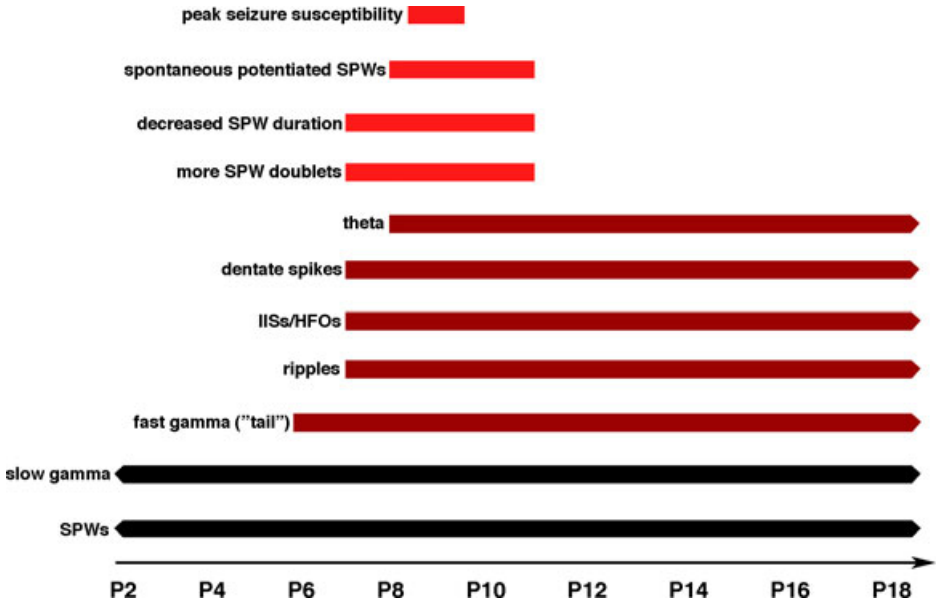

FIG. 7. Summary of results. Schematic presentation of the development of transient and persistent hippocampal events and oscillations in the neonatal rat. SPWs and slow gamma oscillations (20–30 Hz) occurred at all ages examined. SPW-related fast gamma tails (60–100 Hz) and ripples (140–200 Hz) were first detected at P6 and P7, respectively. IISs (IISs) and associated pathological high-frequency oscillations (HFOs; 60–200 Hz) were first evoked by kainic acid infusion into the contralateral hippocampus at P7. Dentate spikes and theta activity first appeared on P7 and P8, respectively. The occurrence of SPW doublets increased and SPW duration decreased transiently between P7 and P11. spSPWs were detected only in P8–11 subjects, and only in a subset of subjects at those ages. Finally, peak seizure susceptibility to kainic acid infusion, as indicated by the number of infusions required to evoke seizure activity, occurred at ~P9.

spikes. In addition, the probability of observing high-frequency gamma, in the form of SPW gamma tails, was highest in those subjects exhibiting spSPWs. It may be significant that AMPA receptors are necessary for the expression of high-frequency gamma oscillations (Mann *et al*., 2005) and that these receptors exhibit their highest level of expression at ~P10 (Sanchez & Jensen, 2001). Moreover, population spikes are most closely associated with highly synchronized hippocampal activity, such as occurs with electrical stimulation of the perforant path and in epileptic adults (Andersen *et al*., 1971; Buzsaki *et al*., 1989, 1991; Leung *et al*., 1995). Thus, spSPWs appear to possess characteristics indicative of increased hippocampal excitability.

It remains unclear how spSPWs fit into the typical developmental trajectory of the hippocampus. Although spSPWs were only observed in P8–11 subjects, not all subjects at these ages exhibited spSPWs. Although it is possible that all infants exhibit spSPWs but differ in the age, duration, and/or probability of their expression, it is also possible that only some infants ever exhibit spSPWs. If the latter is true then early developmental experience, perhaps involving established effects of maternal care on hippocampal development (Meaney, 2001), may determine which infants will express spSPWs. To address these unresolved questions, it will be necessary to study multiple litters and littermates, with and without cross-fostering, during the second postnatal week.

Because we did not examine the effects of KA infusion in all of the initial subjects tested, and because the probability of testing a subject with spSPWs was relatively low, we were not able to determine whether the expression of spSPWs, independent of age, was associated with a greater sensitivity to KA. It is clear, however, that expression of spSPWs is not necessary for rapid induction of seizure activity after KA infusion. Clearly, more work is needed to assess the physiological significance of spSPWs for hyperexcitability and seizure susceptibility.

Kainic acid infusion as an in vivo *test of hyperexcitability*

Having identified the onset of a suite of transient and persistent hippocampal features that together suggest a window of increased excitability, we next sought to establish *in vivo* that seizure activity is most easily induced during this period. Using multiple infusions of KA contralateral to the recording probe, we found that the fewest number of infusions required to produce electrographic seizure activity occurred at ~P9, with decreased efficacy at younger and older ages. The earliest age of seizure induction was P7, coinciding with the developmental onset of SPW-related ripples, as well as decreases in SPW duration and increases in SPW doublets.

The method we developed for inducing seizure activity *in vivo* is based on an *in vitro* method for induction of epileptogenic mirror foci (Khalilov *et al*., 1999, 2003, 2005). This method utilizes the early postnatal development of functional hippocampal commissural projections to induce seizures via contralateral KA infusion; indeed, seizure activity has been shown to propagate to the contralateral hippocampus via the commissures as early as P2 *in vitro* (Khalilov *et al*., 1999). Thus, it appears that the onset of seizure activity at P7 reported here is not attributable to delayed maturation of the hippocampal commissures. Moreover, although our findings appear inconsistent with the reported observation of IISs as early as P2 using the *in vitro* method (Khalilov *et al*., 1999), they are consistent with the finding that epileptogenic high-frequency oscillations (> 60 Hz) are not observed until ~P7 using that same method (Khalilov *et al*., 2005).

Although ipsilateral KA infusions may have been preferable to the contralateral infusions used here, space limitations (i.e. the size of the electrode and syringe in relation to the surface of the infant skull) precluded this approach. It is perhaps for this reason that 'fast ripples' (i.e. 250–600 Hz waveforms), which reliably indicate KA-induced epileptogenesis in adult rats, were not detectable here: such oscillations seem only to be produced after ipsilateral infusions (Bragin *et al*.,

1999b, 2002). Thus, to determine whether the infant hippocampus is capable of generating oscillations within this higher frequency range, ipsilateral infusions would have to be used.

A variety of experimental models have been developed to examine the infantile period of heightened seizure susceptibility, and many are consistent with the results reported here. Specifically, P10–12 marks the period of peak seizure susceptibility when pups are exposed to hypoxia (Jensen *et al*., 1991), P8–11 marks the period of peak seizure response to hyperthermia (Baram *et al*., 1997; Schuchmann *et al*., 2006), and the peak response to high potassium *in vitro* occurs at ∼P11 (Khazipov *et al*., 2004). These peaks also correspond to the period during which the cation–chloride cotransporters NKCC1 and KCC2 are down-regulated and up-regulated, respectively, and GABA thereby begins its transition from excitatory to inhibitory neurotransmission (Rivera *et al*., 1999; Yamada *et al*., 2004).

Conclusion

It is becoming increasingly clear that hippocampal activity in infant rats is characterized by a series of SPW-related phenomena that wax and wane during the early postnatal period, beginning with the startle-SPW relationship at birth (Karlsson *et al*., 2006), its decline at ∼P6 as high-frequency oscillations appear in conjunction with SPWs, and the subsequent partial dissociation of high-frequency activity from SPWs during the third postnatal week. That the expression of spSPWs and other transient features of SPW activity coincide with the down-regulation of NKCC1 and the up-regulation of KCC2 suggests a mechanistic connection between these events. Specifically, if the shift from GABAergic excitation to inhibition is mediated by an activity-dependent mechanism (Ganguly *et al*., 2001; Fiumelli & Woodin, 2007) then perhaps the increased activity associated with hyperexcitability promotes a self-limiting process by which the infant transitions to a nonhyperexcitable state.

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Abbreviations

CA1, cornu ammonis 1; CA3, cornu ammonis 3; CSD, current source density; EEG, electroencephalogram; EMG, electromyogram; IIS, interictal spike; KA, kainic acid; LFP, local field potential; P, postnatal day; spSPW, spontaneous potentiated SPW; SPW, sharp wave.

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