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Transient epileptiform signaling during neuronal network development: regulation by external stimulation and bimodal GABAergic activity

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ABSTRACT

A predominance of excitatory activity, with protracted appearance of inhibitory activity, accompanies cortical neuronal development. It is unclear whether or not inhibitory neuronal activity is solicited exclusively by excitatory neurons or whether the transient excitatory activity displayed by developing GABAergic neurons contributes to an excitatory threshold that fosters their conversion to inhibitory activity. We addressed this possibility by culturing murine embryonic neurons on multi-electrode arrays. A wave of individual 0.2–0.4 mV signals ("spikes") appeared between approx. 20–30 days in culture, then declined. A transient wave of high amplitude (>0.5 mV) epileptiform activity coincided with the developmental decline in spikes. Bursts (clusters of ≥3 low-amplitude spikes within 0.7 s prior to returning to baseline) persisted following this decline.

Addition of the GABAergic antagonist bicuculline initially had no effect on signaling, consistent with delayed development of GABAergic synapses. This was followed by a period in which bicuculline inhibited overall signaling, confirming that GABAergic neurons initially display excitatory activity in *ex vivo* networks. Following the transient developmental wave of epileptiform signaling, bicuculline induced a resurgence of epileptiform signaling, indicating that GABAergic neurons at this point displayed inhibitory activity. The appearance of transition after the developmental and decline of epileptiform activity, rather than immediately after the developmental decline in lower-amplitude spikes, suggests that the initial excitatory activity of GABAergic neurons contributes to their transition into inhibitory neurons, and that inhibitory GABAergic activity is essential for network development.

Prior studies indicate that a minority (25%) of neurons in these cultures were GABAergic, suggesting that inhibitory neurons regulate multiple excitatory neurons. A similar robust increase in signaling following cessation of inhibitory activity in an artificial neural network containing 20% inhibitory neurons supported this conclusion. Even a minor perturbation in GABAergic function may therefore foster initiation and/or amplification of seizure activity, as well as perturbations in long-term potentiation.

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1. Introduction

Normal brain function requires a balance of excitatory and inhibitory signaling, perturbation in which can lead to seizures (Blumenfeld, 2005; Chiappalone et al., 2009; Förstera et al., 2010; Lopes da Silva et al., 2003; McCormick and Contreras, 2001). While seizures initiate from the activity of individual neurons (Miles and Wong, 1983; Timofeev and Steriade, 2004), whether or not a seizure manifests is dependent upon amplification of this initiating signal. Amplification encompasses feedback to the region of onset, as well as synaptic spread to adjacent regions, both of which recruit additional neurons (Coulter and DeLorenzo, 1999; Lado and Moshe, 2008; MacVicar and Dudek, 1980; Miles and Wong, 1983; Rutecki et al., 1989). What prevents or terminates seizures is unfortunately not completely resolved [for review, see Lado and Moshe, 2008].

Inhibitory neurons are thought to play important roles in seizure termination and prevention/reduction of spreading. Inhibition is mediated at least in part by release of the neurotransmitter gamma aminobutyric acid (GABA; *e.g.*, Bernard et al., 2000). Seizures normally also generate inhibitory signals by feedback within the initiation zone (Dorn and Witte, 1995; Kostopoulos et al., 1983), which can induce pauses in excitatory firing. Conversely, since inhibitory neurons connect with multiple excitatory neurons, synchronization of excitatory signaling can under some conditions promote epileptiform conditions (Dugladze et al., 2007). Since feedback signaling recruits additional excitatory and inhibitory neuronal activity, avoidance/termination of seizures likely depends upon a critical balance of excitatory and inhibitory neurons either

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locally and/or among brain regions (Timofeev and Steriade, 2004), as does maintenance of normal signaling patterns (Zhang and Sun, 2011). In this regard, inhibitory neuronal activity, including that mediated by GABA, also affects long-term excitability (Brooks-Kayal, 2005), and therefore likely restricts spontaneous signaling in general.

Seizures can promote excitotoxic depletion of inhibitory neurons (Dinocourt et al., 2003); resultant depletion of inhibitory activity will likely promote increased seizure incidence and duration. In addition, increased glutamergic sprouting that can accompany excitatory bursts in developing hippocampus during temporal lobe epilepsy (Esclapez et al., 1999) also promotes seizure activity. Enhancement of GABAergic activity to maintain an appropriate balance of excitatory and inhibitory activity therefore represents a potential therapeutic direction to reduce seizures [for review, see Lado and Moshe, 2008].

Excitatory synaptic connections are prominent early in development, and are followed by the establishment of functional inhibitory activity (Eichler and Meier, 2008; Wake et al., 2007). This differential establishment promotes synaptic plasticity but also allows a developmental window of increased seizure activity (Briggs and Galanopoulou, 2011; Esclapez et al., 1999; Luhmann and Prince, 1991; Sutor and Luhmann, 1995). Developmental deficiencies or delays in recruitment of inhibitory neurons may promote epileptic disorders (Briggs and Galanopoulou, 2011). Inhibitory neuronal activity may be solicited by progressively increasing excitatory activity (Marty et al., 2000; Seil, 1999; Spitzer, 2006; Zhang and Poo, 2001). Moreover, some studies suggest that GABAergic neurons themselves are initially excitatory and then progressively display inhibitory activity (Ben-Ari, 2002; Noh et al., 2010; Owens and Kriegstein, 2002; Takayama and Inoue, 2004). Regardless of which (or both) event(s) is/are responsible, timely establishment of inhibitory activity is essential for nervous system development.

Neuronal networks established on micro-electrode arrays (MEAs) provide useful models for some of these phenomena, since they develop functional networks that transmit synaptic signals over considerable distances (Bakkum et al., 2008; Brewer et al., 2009; Chao et al., 2005; Jimbo et al., 1998; Madhavan et al., 2005; Van Pelt et al., 2004a, 2004b; Wagenaar et al., 2004; Zemianek et al., 2012a, 2012b). Neurons display spontaneous signals shortly after plating. Over the first few weeks in culture, individual signals predominate, and several laboratories have reported periods of relatively high-amplitude epileptiform spikes in developing networks. Long-term cultures (*i.e.*, those maintained for ≥1–2 months) develop spontaneous, complex signals (termed "bursts" by convention) (Madhavan et al., 2007; Rolston et al., 2007; Wagenaar et al., 2006; Zemianek et al., 2012a) that resemble the bursting activity observed during development *in situ* (Ben-Ari, 2001; Khazipov et al., 2004; Zhang and Poo, 2001).

GABAergic neurons constitute a minority (approx. 25%) of the population in embryonic cortical cultures (Serra et al., 2010), and formation of inhibitory synapses in cultures parallels the delay observed *in situ* (Burgard and Hablitz, 1993; Ichikawa et al., 1993; Luhmann and Prince, 1991). The establishment of the mature signal pattern is dependent upon inhibitory neuronal activity, since relatively sparse cultures do not develop a mature signaling pattern but instead continue to display individual spikes of relatively high amplitude due to insufficient inhibitory activity (Ito et al., 2010; Serra et al., 2010). Furthermore, treatment of dense, mature cultures with GABAA receptor antagonist bicuculline results in reversible resumption of epileptiform signaling in dense, mature cultures (Serra et al., 2010). Treatment with glutamate also induces epileptiform activity (Srinivas et al., 2007). A progressive degradation of mature signal patterns accompanies the selectively loss of inhibitory neurons as cultures age (Li et al., 2007). These findings collectively indicate that the so-called mature bursts characteristic of long-term cultures (Kamioka et al., 1996; Madhavan et al., 2007; Rolston et al., 2007; Wagenaar et al., 2006) may be derived at least in part by modulation of excitatory activity (Serra et al., 2010).

We recently demonstrated that organized bursts appeared relatively early during *ex vivo* network development, while individual spikes predominated. Subsequently, the amount of individual spikes declined, while a similar amount of bursts continued, yielding a relative, rather than absolute, increase in bursts (Zemianek et al., 2012a). The so-called mature pattern, in which bursts predominate, may reflect progressive loss of aberrant signal activity, consistent with continued establishment as well as pruning of synaptic connections (Wu et al., 2012; Zemianek et al., 2012a). This possibility was supported by hastening of the transition to a mature signal pattern by external stimulation of developing networks with a synaptic signal (Zemianek et al., 2012a). Herein, we monitored the role of GABAergic neurons in the onset and cessation of the transient period of epileptiform activity during *ex vivo* network development.

2. Materials and methods

2.1. Generation of embryonic cultures

Primary murine embryonic cortical neurons harvested at day 17 of gestation from C57BL/6 mice were plated at ≥100 cells/mm^2 on poly-D-lysine/fibronectin-coated, MEAs (Multichannel Systems, Reutlingen, Germany) in B27-supplemented Neurobasal medium (Invitrogen, Carlsbad, CA) as described (Serra et al., 2008). Sacrifice of the pregnant female was according to procedures approved by our Institutional Animal Care and Use Committee. Cultures were plated at a density corresponding to "dense" cultures (Fig. 1A and B; see Serra et al., 2010). In efforts to achieve a natural environment relative to *in situ* conditions, the chamber surface was coated with a 50–50% mixture of poly-L-lysine/fibronectin, and we made no efforts to eliminate glial cells, which may contribute to synaptic development (Bolton and Eroglu, 2009; Serra et al., 2010). This fostered astroglial proliferation which resulted in a confluent astroglial monolayer within 10–14 days of culture.

2.2. Recording and stimulation

MEAs were placed in a MEA-1060-INV amplifier (Multichannel) and synaptic activity recorded *via* a DT9814 data acquisition system (Data Translation; Marlborough, MA). Data files (30 s in length) for each channel were analyzed manually and with an algorithm that distinguishes bona-fide neuronal activity from baseline electrical disturbances (Wagenaar et al., 2005). Prior studies using combinations of excitatory and inhibitory neuronal antagonists have confirmed synaptic origin of signals (Serra et al., 2010). Signals were classified as spikes (individual signals 0.2–0.4 mV in amplitude separated by other signals by an minimum of 0.7 s), epileptiform (spikes >0.5 mV in amplitude), or bursts (clusters of ≥3 spikes within 0.7 s prior to returning to baseline) (Madhavan et al., 2007; Rolston et al., 2007; Wagenaar et al., 2006; Zemianek et al., 2012a).

Developing networks were stimulated with a 1 s segment of a spontaneous signal recorded from a mature culture, previously demonstrated to invoke robust synaptic responses (Zemianek et al., 2012a, 2012b). This signal was applied at a singular MEA channel with an adjacent electrode utilized as a ground, which restricted spread of the signal among synaptically connected clusters of neurons (Zemianek et al., 2012b). Cultures were stimulated and recorded every 2nd day for 28 days commencing at day 15 (prior to appearance of robust spontaneous signals). Signals were quantified before and after the addition of bicuculline (5 μM; Serra et al., 2010).

2.3. Design of artificial neural network

To model the potential impact of inhibitory neurons on excitatory neuronal activity in mature networks, we created a simulated neuronal network using the "Brian" neural network simulation framework. The network consisted of 135 simulated leaky integrate-and-fire spiking neurons. The connections between the neurons were configured by a script which simulates the plating of neurons on a MEA and their eventual growth and wiring. The neurons connect to each other based on a Gaussian distribution model for the probability of a pair of cells to connect based on the straight-line distance between them. The parameters of the Gaussian distribution are set to maximize connectivity around 200 μm from the cell body. This model and its parameters are based on the simplified chemotactic model of Kahng et al. (2007) and Segev and Ben-Jacob (2000). The inhibitory weight of the neurons initially set to −9 mV and overall network activity was recorded. The inhibitory weight was then set to 0 mV (*i.e.*, such that inhibitory neurons had no effect) and activity was again recorded.

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