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Metabolic responses differentiate between interictal, ictal and persistent epileptiform activity in intact, immature hippocampus *in vitro*☆

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ABSTRACT

Interictal spikes, ictal responses, and status epilepticus are characteristic of abnormal neuronal activity in epilepsy. Since these events may involve different energy requirements, we evaluated metabolic function (assessed by simultaneous NADH and FAD+ imaging and tissue O2 recordings) in the immature, intact mouse hippocampus (P5-P7, *in vitro*) during spontaneous interictal spikes and ictal-like events (ILEs), induced by increased neuronal network excitability with either low Mg2 + media or decreased inhibition with bicuculline. In low Mg2 + medium NADH fluorescence showed a small decrease both during the interictal build-up leading to an ictal event and before ILE occurrences, but a large positive response during and after ILEs (up to 10% net change). Tissue O2 recordings (pO2) showed an oxygen dip (indicating oxygen consumption) coincident with each ILE at P5 and P7, closely matching an NADH fluorescence increase, indicating a large surge in oxidative metabolism. The ILE O2 dip was significantly larger at P7 as compared to P5 suggesting a higher metabolic response at P7. After several ILEs at P7, continuous, low voltage activity (late recurrent discharges: LRDs) occurred. During LRDs, whilst the epileptiform activity was relatively small (low voltage synchronous activity) oxygen levels remained low and NADH fluorescence elevated, indicating persistent oxygen utilization and maintained high metabolic demand. In bicuculline, NADH fluorescence levels decreased prior to the onset of epileptiform activity, followed by a slow positive phase, which persisted during interictal responses. Metabolic responses can thus differentiate between interictal, ictal-like and persistent epileptiform activity resembling status epilepticus, and confirm that spreading depression did not occur. These results demonstrate clear translational value to the understanding of metabolic requirements during epileptic conditions.

Introduction

Epileptogenic brain regions display altered metabolism associated with structural damage, cell loss, or changes in vascular supply (Pan et al., 2008; Alavi et al., 2011). The increased energy demand associated with ictal activity is usually matched with an appropriate metabolic response (Duffy et al., 1975; Chapman et al., 1977; Folbergrova et al., 1985). For example, fluorodeoxyglucose (FDG) PET scans in humans show basal hypometabolic regions between seizure events, which convert to hypermetabolic conditions during and after a seizure (Pan et al., 2008; Alavi et al., 2011). The metabolic demands associated with interictal and ictal epileptiform events can be estimated in animal models using fluorescent FAD + or NADH metabolic recordings (O'Connor et al., 1972; Schuchmann et al., 1999; Kovacs et al., 2001; Heinemann et al., 2002a, 2002b; Kann et al., 2005; Dora, 1983; Cooper et al., 2009; Zhao et al., 2011), or cytochrome analysis with oxygen recordings (Kreisman et al., 1983). Additionally, the spread of ictal events can be estimated with intrinsic optical signal changes associated with cell swelling (Holtkamp et al., 2011). Thus, estimates of metabolic demand, particularly NADH/FAD + imaging and tissue oxygen levels, may help differentiate epileptiform states in an exposed brain (i.e., surgical exposure), the counterpart of FDG and oxygen uptake PET scans in a closed preparation and under clinical conditions.

Whereas both cytosolic and mitochondrial pathways generate the critical metabolic co-factor NADH (nicotinamide adenine nucleotide), metabolic $FADH_2$ pathways and O_2 consumption primarily occur within mitochondria (Foster et al., 2005; McKenna et al., 2006; Shetty et al., 2012). Fluorescence of either NADH (at 460 nm) (Aubin, 1979; Kasischke et al., 2011) or FAD + (at 515 nm) (Zhao

Abbreviations: NADH, nicotine adenine dinucleotide; FAD +, flavin adenine nucleotide; ILEs, ictal-like events; LRD, late recurrent depolarization; aCSF, artificial cerebrospinal fluid; LFP, local field potential

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et al., 2011) can be used to monitor energy metabolism (Foster et al., 2005; Galeffi et al., 2007; Galeffi et al., 2011). NADH fluorescence levels also indirectly reflect oxygen availability to a critical inflection point (near 7.5 mm Hg O2), below which most NAD + is converted to NADH (Erecinska and Silver, 2001; Galeffi et al., 2011; Kasischke et al., 2011). Hence, NADH imaging and direct O2 tissue levels together provide an integrated estimate of neuronal metabolism. For example, in *in vitro* brain preparations (i.e., with no circulatory perfusion or hemodynamic response but with constant oxygen diffusion into the tissue) increased oxygen demand in the tissue leads to a lowered oxygen tension (Foster et al., 2005; Galeffi et al., 2007; Galeffi et al., 2011). Conversely, if oxygen demand is reduced (i.e., from cell death), the tissue oxygen tension can rise above baseline tissue levels (Foster et al., 2005). Depending on oxygen availability, heightened oxygen demand and utilization may be associated with either an NADH fluorescence decrease (i.e., a decreased NADH/NAD + ratio) or an increase (Turner et al., 2007; Galeffi et al., 2011; Shetty et al., 2014). Thus, tissue oxygen recordings in concert with NADH/FAD + imaging can reveal the level of ongoing oxidative metabolism. However, the pattern of oxygen responses differs significantly between intact (Kreisman et al., 1981) and *in vitro* preparations (Foster et al., 2005) because the delivery of oxygen is different (active transport by blood circulation vs passive diffusion from artificial cerebrospinal fluid, respectively).

Seizure-like events (SLEs) can be induced *in vitro* with a variety of procedures, including nominally zero Mg^{2+} conditions (Mody et al., 1987; Dreier and Heinemann, 1991; Gloveli et al., 1995), GABA-A antagonists (Cooper et al., 2009), and voltage gated potassium channel blockers [ie, 4-aminopyridine] (Schuchmann et al., 1999; Kibler and Durand, 2011). These models of epileptiform activity display interictal-like events (IEs), SLEs (Gloveli et al., 1995; Heinemann et al., 2002a, 2002b), and late recurrent discharges (LRDs) reminiscent of status epilepticus (Dreier and Heinemann, 1991; Schuchmann et al., 1999). Such patterns are similar to those induced *in vivo* (Chapman et al., 1977; Dora, 1983; Folbergrova et al., 1985). The intact, immature hippocampus *(in vitro)* also demonstrates IEs, SLEs and LRDs in zero Mg2 + conditions (Quilichini et al., 2002; Quilichini et al., 2003; Dzhala et al., 2010; Quilichini et al., 2012). Clarifying energy metabolism during these various activities may provide a framework for the design of treatments which could prevent seizure-induced damage (Chen et al., 2007). We have used the intact immature hippocampus preparation *in vitro* for these studies because it preserves the hippocampal circuitry compared to brain slices (Schwartzkroin, 1986), whilst remaining easily amenable to imaging and electrophysiological recordings.

Our hypotheses center on metabolic conditions underlying these epileptiform events, particularly that metabolic differences in tissue maturity underlay the transition to late recurrent depolarizations (LRDs) in P7 (more mature) hippocampus, that LRD occurrence is a nonconvulsive status response rather than a spreading depression response, and that there are critical differences in the metabolic responses to bicuculline (with disinhibition) and low Mg2 + conditions (with enhanced excitatory drive, particularly NMDA-mediated). To assess these hypotheses we analyzed energy metabolism (assessed by NADH/FAD + fluorescence and tissue oxygen levels) during epileptiform events induced in low Mg2 + conditions or following blockade of fast GABAergic transmission with bicuculline. We show that metabolic activity and a moderate decrease in NADH fluorescence can precede synchronous discharge, whereas a rise in NADH tissue fluorescence and significant oxygen utilization occur together during and after epileptiform events, indicating a high level of metabolic demand. Both IE and ILE activities are similar in zero Mg2 + and bicuculline conditions with only differences present in the oxidative phase, indicating that neuronal synchrony leads to intense metabolic demand. However, during LRDs in zero Mg2 + conditions NADH tissue fluorescence and oxygen utilization are considerably increased, to maintain low voltage but highly synchronized electrical activity, consistent with nonconvulsive status epilepticus.

Experimental procedures

Immature hippocampus in vitro preparation

All protocols were designed and approved according to INSERM and international guidelines for experimental animal care and use. Experiments were performed on intact hippocampi taken from FVB NG mice between postnatal (P) day 5 and 7 (P0 was day of birth) (Quilichini et al., 2002; Quilichini et al., 2012). The immature mice were decapitated rapidly after being cooled on ice as a temporary anaesthesia. The brain was extracted from the skull and transferred to oxygenated (95% O2 / 5% CO2) ice cold (4 °C) artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 126; KCl 3.5; CaCl2 2; MgCl2 1.3; NaHCO3 25; NaHPO4 1.2; glucose 10 (pH = 7.3). After at least 2 hours incubation at room temperature in aCSF, hippocampi were transferred to the recording chamber. To ensure a high perfusion rate we used a closed circuit perfusion system with recycling: 250 ml of solution were used per hippocampus and per condition. The pH (7.3) and temperature (33 °C) were controlled during all experiments.

Induction of epileptiform events

Bicuculline (10 μM) was added to the ACSF after a 30 min baseline period, which generated both spontaneous interictal and ictal-like epileptiform responses (Cooper et al., 2009). In contrast, low Mg2 + media (zero added Mg2 +) leads to more prolonged periods of hyperexcitability (ie, 60–90 sec) (Mody et al., 1987; Dreier and Heinemann, 1991; Gloveli et al., 1995). After 30 min baseline recording in Mg2 + containing aCSF solution, the media was switched to one without added Mg2 + ion. In this condition, the extracellular concentration of Mg2 + may be influenced by other constituents of the aCSF, possibly near 0.08 mM (Mody et al., 1987). Therefore, we use the term low-Mg2 + aCSF rather than zero-Mg2 + aCSF. Because Mg2 + is a biologically active molecule for both membrane stability and enzyme activation (Altura and Altura, 1996), the bicuculline condition provides an alternative mechanism for studying epileptiform activity and neuronal synchronization.

Physiological monitoring

Extracellular glass electrodes were placed into the mid CA1 region in each hippocampus, filled with low Mg2 + aCSF. The intact immature hippocampi were positioned within the chamber so that the CA1 area was up and the DG region down; the CA1 area was superficial and accessible for the recording electrodes and imaging using either transmission or epifluorescence. Local field potentials (LFPs; either spontaneous or evoked) were amplified with DAM-80 differential amplifiers (WPI) for AC-coupled recordings and a MultiClamp700B amplifier for DC-coupled recording, then digitized with a Digidata 1200B (Axon Instruments, Molecular Devices), stored on the hard drive of the personal computer and analyzed using PClamp 8.2 software (Molecular Devices). The extracellular spontaneous field potential was observed for epileptiform events.

NADH/FAD + fluorescence imaging

NADH autofluorescence for large-scale imaging was measured using UV light emitted by a 300 W pre-centered mercury lamp (Nikon Intensilight C-HGFI) passed through a fiber-optic cable and a 290–370 nm excitation filter. After trying several configurations of transmitted and refracted light angles a direct transmitted approach through the bottom of the tissue gave the best signal to noise ratio, with recordings from above the tissue using either a dissecting microscope or a lens mounted directly on the camera with the emission filter (long pass > 420 nm), due to the large field size needed to monitor the whole hippocampi (~12 mm). This transmitted approach effectively averaged the NADH signal across the entire tissue depth of the intact hippocampus (~1 mm). The emitted light was captured using a linear, cooled 12 bit CCD camera (PCO VGA Sensicam, Germany) equipped with 420 nm long pass filter