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METHYLXANTHINE-EVOKED PERTURBATION OF SPONTANEOUS AND EVOKED ACTIVITIES IN ISOLATED NEWBORN RAT HIPPOCAMPAL NETWORKS

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Abstract—Treatment of apnea of prematurity with methylxanthines like caffeine, aminophylline or theophylline can evoke hippocampal seizures. However, it is unknown at which interstitial brain concentrations methylxanthines promote such neonatal seizures or interfere with physiological 'early network oscillations' (ENOs) that are considered as pivotal for maturation of hippocampal neural networks. We studied theophylline and caffeine effects on ENOs in CA3 neurons (CA3-ENOs) and CA3 electrical stimulation-evoked monosynaptic CA1 field potentials (CA1-FPs) in sliced and intact hippocampi, respectively, from 8 to 10-days-old rats. Submillimolar doses of theophylline and caffeine, blocking adenosine receptors and phosphodiesterase-4 (PDE4), did not affect CA3-ENOs, ENO-associated cytosolic Ca^{2+} transients or CA1-FPs nor did they provoke seizure-like discharges. Low millimolar doses of theophylline (⩾1 mM) or caffeine (⩾5 mM), blocking $GABA_A$ and glycine receptors plus sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA)-type Ca^{2+} ATPases, evoked seizure-like discharges with no indication of cytosolic Ca^{2+} dysregulation. Inhibiting PDE4 with rolipram or glycine receptors with strychnine had no effect on CA3-ENOs and did not occlude seizure-like events as tested with theophylline. $GABA_A$ receptor blockade induced seizure-like discharges and occluded theophylline-evoked seizure-like discharges in the slices, but not in the intact hippocampi. In summary, submillimolar methylxanthine concentrations do not acutely affect spontaneous CA3-ENOs or electrically evoked synaptic activities and low millimolar doses are needed to evoke seizure-like discharges in isolated developing hippocampal neural networks. We conclude that mechanisms of methylxanthine-related seizure-like discharges do not involve SERCA inhibition-related neuronal Ca^{2+} dysregulation, PDE4 blockade or adenosine and glycine receptor inhibition, whereas $GABA_A$ receptor blockade may contribute partially.

Key words: calcium imaging, cortical networks, epilepsy, hippocampus, methylxanthine, neural network bursting.

INTRODUCTION

Methylxanthines like caffeine, aminophylline or theophylline are the gold standard for countering apnea of prematurity, but convulsive seizures can occur as a serious side effect (Delanty et al., 1998; Bhatia, 2000; Korematsu et al., 2008; El-Bitar and Boustany, 2009). Such seizures originate from cortico-hippocampal networks that are yet immature at birth (Khazipov and Luhmann, 2006; Ben-Ari et al., 2007; Sipilä and Kaila, 2008; Rakic, 2009). These neural networks show spontaneous discharges, referred to as early network oscillations (ENOs), spindle bursts or gamma oscillations, that are considered as pivotal to consolidate synaptic connectivity, particularly via the associated depolarization-related rises of cytosolic Ca^{2+} (Goodman and Shatz, 1993; Leinekugel et al., 1997; Canepari et al., 2000; Garaschuk et al., 2000; Ben-Ari et al., 2007; Sipilä and Kaila, 2008; Yang et al., 2009, 2013).

In preterm infants, methylxanthines cause seizures predominantly at blood plasma levels >50 μM (Delanty et al., 1998; Comer et al., 2001; Korematsu et al., 2008; El-Bitar and Boustany, 2009). Although this value is higher than that for impeding adenosine receptors, several of the latter studies consider blockade of tonic neural network inhibition by endogenous adenosine as a major mechanism of such seizures. These reports also point out that therapeutic plasma methylxanthine levels can be as high as 500 μM (Fredholm et al., 1999). As this dose blocks phosphodiesterase-4 (PDE4) (Fredholm et al., 1999), the resulting increase of cellular cyclic-adenosine monophosphate (cAMP) levels may also be epileptogenic. At 1–10 mM, methylxanthines inhibit both A-type γ-aminobutyric acid ($GABA_A$) and glycine receptors as well as sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPases (SERCAs) (Fredholm et al., 1999). These mechanisms might also play a role in seizures, if methylxanthines accumulate in brain interstitial space to doses higher than in plasma (Ruangkittisakul and Ballanyi, 2010; Panaitescu et al., 2013).

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Abbreviations: CA1-FPs, CA1 field potentials; cAMP, cyclic-adenosine monophosphate; ENOs, early network oscillations; preBötC, pre-Bötzinger Complex; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase.

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The main objective of our study was to determine threshold concentrations of the frequently used methylxanthines caffeine and theophylline for evoking seizure-like activities in neonatal hippocampal networks and analyze the underlying mechanisms. As a further objective, we studied if these agents affect hippocampal ENOs at doses close to, and slightly above, the common upper limit for therapeutic plasma levels, i.e. 100 and 500 μM, respectively. We also applied low millimolar methylxanthine to assess effects on spontaneous and evoked hippocampal activities caused by $GABA_A$/glycine receptor and SERCA inhibition. For this, we recorded ENOs in the CA3 area (CA3-ENOs) of hippocampal slices (Ben-Ari et al., 1989, 2007; Sipilä and Kaila, 2008) and electrically evoked CA1 field potentials (CA1-FPs) in intact isolated hippocampi (Khalilov et al., 1997; Kilb et al., 2007) from neonatal rats. In the slices, these electrophysiological population recordings were complemented by imaging of dynamic changes of the free cytosolic Ca^{2+} concentration in groups of visualized CA3 neurons using multiphoton microscopy (Ruangkittisakul et al., 2008). One aspect of such Ca^{2+} imaging was to study whether methylxanthine-evoked seizures induce SERCA inhibition-related acute Ca^{2+} dysregulation that initiates the death of hippocampal neurons in other types of seizures (Griffiths et al., 1984; Pal et al., 2000).

EXPERIMENTAL PROCEDURES

ENOs originate from CA3 neurons and trigger synchronous discharges in other hippocampal areas including CA1 (Ben-Ari et al., 2007; Sipilä and Kaila, 2008). Here, we studied methylxanthine effects on CA3-ENOs in slices (Kantor et al., 2012) and monosynaptic CA1-FPs evoked by electrical CA3 stimulation in intact hippocampi which lack spontaneous activity, but comprise an intact neural circuitry (Khalilov et al., 1997; Luhmann and Kilb, 2012). Both *in vitro* models were isolated from 8 to 10-days-old rats to reflect hippocampal development in newborn human infants (Alling, 1985; Clancy et al., 2001; Rakic, 2009). Experiments on CA3-ENOs were performed in Edmonton in compliance with guidelines of the 'Canadian Council for Animal Care' and with approval of the University of Alberta Health Animal Care and the 'Use Committee for Health Sciences'. Experiments on CA1-FPs in Mainz were conducted in accordance with EU directive 86/609/EEC for use of animals in research and the 'NIH Guide for the Care and Use of laboratory animals' and were approved by the local ethics committee (Landesuntersuchungsanstalt RLP, Koblenz, Germany). All efforts were made to reduce the number of experimental animals and their suffering.

Solutions and drugs

Superfusate contained (in mM) 120 NaCl, 3 (intact hippocampi) or 4 KCl (slices), 1.2 $CaCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10–20 D-glucose; pH adjusted to 7.4 by gassing with carbogen (95% O_2, 5% CO_2). Slices were generated in ice-cold solution comprising (in mM) 4 KCl, 234 sucrose, 8 $MgSO_4$, 26 $NaCO_3$, 1 NaH_2PO_4, and 10 D-glucose, pH adjusted to 7.4 by carbogenation. Superfusate contained 3 mM K^+ for isolating and studying hippocampi, whereas slices were generated, stored and studied in 4 mM K^+ to obtain more stable CA3-ENOs (Kantor et al., 2012). All drugs were bath-applied. Theophylline or caffeine (both from Sigma–Aldrich) were tested at increasing doses (0.1, 0.25, 0.5, 1, 2.5, 5 and 10 mM) for 10–20 min followed by washout for 15–30 min until effects reversed. $GABA_A$ receptors were blocked with gabazine (10 mM stock in H_2O; Tocris Biosciences, Ellisville, MO, USA) and glycine receptors with strychnine (10 mM stock in H_2O; Sigma). Rolipram (10 mM stock in DMSO; Sigma) was applied to inhibit PDE4 (Spina, 2008; Barad et al., 1998; Ruangkittisakul and Ballanyi, 2006, 2010). Fluo-4-AM (Invitrogen, Carlsbad, CA, USA) was used for population cytosolic Ca^{2+} imaging (5 mM stock in DMSO + 20% pluronic acid).

Preparations and electrophysiological recording

Horizontal slices: Slice preparation and electrophysiological plus Ca^{2+} imaging approaches are described in detail elsewhere (Kantor et al., 2012). In brief, Sprague–Dawley or Wistar rat pups were anesthetized with isoflurane vapor until the paw withdrawal reflex disappeared. They were then decapitated and their brain isolated at 0–4 °C in the preparation solution and glued ventral side down to the vise of a VT1000S vibratome (Leica Biosystems, Richmond Hill, ON, Canada) for cutting 400–500-μm-thick horizontal sections in anterior to posterior direction. Slices were stored for at least 1 h at 25–27 °C in standard solution on a nylon net in a beaker that was positioned in a water bath (Isotemp-102S, Fisher, Canada). For experiments, slices were either fixed in a submerged-type acrylic chamber with insect pins on a silicone layer (for CA3-ENO recording) or a metal harp with nylon threads (for Ca^{2+} imaging). Superfusate was applied at a flow rate of ~5 ml/min via a peristaltic pump (Watson-Marlow Alitea-AB; Sin-Can, Calgary, AB, Canada) and kept at 30–31 °C in the recording chamber (TC-324B; Harvard Apparatus, Saint-Laurent, QE, Canada). Extracellular activity was recorded with glass suction electrodes (GC-150-TF-10, Harvard Apparatus; outer tip ø 50–120 μm) positioned on the slice surface in the CA3 area. Signals were amplified (x10 k), bandpass-filtered (0.3–3 kHz), AC-recorded (Model 1700, AM Systems, Sequim, WA, USA), integrated (τ: 20–50 ms; CWE Inc., Ardmore, PA, USA) and sampled at a rate of 1–4 kHz (Powerlab/8SP; ADInstruments, Colorado Springs, CO, USA). Changes in the baseline of the integrated signals indicate alterations in spontaneous discharge in populations of neurons (Ruangkittisakul and Ballanyi, 2010; Panaitescu et al., 2013). In a single slice, 3–7 methylxanthine concentrations were tested.

Intact hippocampi: hippocampi were isolated *en bloc* as described before (Moser et al., 2006; Kilb et al., 2007; Luhmann and Kilb, 2012). In short, Wistar rat pups were anesthetized with enflurane (Ethrane, Abbot Wiesbaden, Germany), decapitated and their brains isolated and