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Chronic exposure to alcohol alters network activity and morphology of cultured hippocampal neurons

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ABSTRACT

The effects of chronic exposure to moderate concentrations of ethanol were studied in cultured hippocampal neurons. Network activity, assessed by imaging of $[Ca^{2+}]i$ variations, was markedly suppressed following 5 days of exposure to 0.25–1% ethanol. The reduced activity was sustained following extensive washout of ethanol, but the activity recovered by blockade of inhibition with bicuculline. This reduction of network activity was associated with a reduction in rates of mEPSCs, but not in a change in inhibitory synaptic activity. Chronic exposure to ethanol caused a significant reduction in the density of mature dendritic spines, without an effect on dendritic length or arborization. These results indicate that chronic exposure to ethanol causes a reduction in excitatory network drive in hippocampal neurons adding another dimension to the chronic effects of alcohol abuse.

1. Introduction

Alcohol addiction is a major medical burden in modern world as it affects large populations across the world, is not prohibited by law, and has severe consequences to quality and longevity of human life. Numerous studies have identified primary molecular targets for acute alcohol action in the brain, including, but not restricted to potassium channels (Li et al., 2013; Bukiya et al., 2014), Glutamate and GABAergic synapses (Santhakumar et al., 2007; Zorumski et al., 2014) and synaptic scaffold proteins (Romero et al., 2010, 2013). However, the neural basis of the long term effect of alcohol is not entirely clear. Using dissociated cultures of central neurons, a diversity of somewhat contradictory morphological and chemical consequences of chronic exposure to ethanol have been reported. These include neuronal cell death (Pickering et al., 2010), a reduction in dendritic spine density and maturity (Romero et al., 2013), or an increase in dendritic spine size associated with an increase in the density of NMDA receptor clusters (Carpenter-Hyland and Chandler, 2006). The diversity of effects may reflect different ethanol concentrations, duration of exposure and age of onset of the chronic treatment, as well as differences in growth conditions and source of the tested tissue (dissociated culture vs cultured slice). In addition, few if any studies combined observations on the development of changes in activity of the tested neurons with the morphological observations. This is an important issue if we are to identify the primary and/or the secondary effects of chronic exposure to ethanol. In fact, in only a handful of studies the effects of chronic exposure to ethanol on electrical activity of the exposed tissue have been reported (Tu et al., 2007; Yool and Gruol, 1987), unlike the case with acute exposure (e.g. Basavarajappa et al., 2008; Brodie et al., 2007). In addition, the timing of exposure to ethanol is important if we are to conclude whether the drug affected developing neurons or also already mature ones. In the present study we exposed cultured hippocampal neurons to different concentrations of ethanol, for different durations, at different ages in vitro, and used a calcium sensitive method for detecting and analyzing spontaneous activity of the cultured neuronal networks. We wish to report that ethanol exposure exerts a complex, time and concentration dependent action on the morphology and function of these neurons.

Abbreviation: mEPSC, miniature excitatory postsynaptic current.

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2. Methods

2.1. Hippocampal culture

Animal handling was done in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Weizmann Institute of Science, and the appropriate Israeli law. Cultures were prepared as detailed elsewhere (Korkotian and Segal, 1997). Briefly, rat pups were decapitated on day of birth (P0), their brains removed and placed in a chilled (4 °C), oxygenated Leibovitz L15 medium (Gibco) enriched with 0.6% glucose and gentamicin (Sigma, 20 μg/ml). Hippocampal tissue was mechanically dissociated after incubation with trypsin (0.25%) and DNAase (50 μg/ml), and passed to the plating medium consisting of 5% heat-inactivated horse serum (HS), 5% fetal calf serum, and B-27 (1 μl/1 ml) prepared in minimum essential medium (MEM) Earl salts (Gibco), enriched with 0.6% glucose, gentamicin (20 μg/ml), and 2 mM glutamax (enriched MEM). Cells were suspended in 1 ml medium and plated on 12 mm round cover glasses in 24-well plates. Cells were left to grow in the incubator at 37 °C, 5% CO_2 for 4 days, at which time the medium was changed to 10% HS in enriched MEM, plus a mixture of 5′-fluoro-2-deoxyuridine/uridine (FUDR) (Sigma, 20 μg and 50 μg/ml, respectively), to block glial proliferation. Four days later the medium was replaced by 10% HS in MEM.

2.2. Chronic treatment of cultures with ethanol

Ethanol was added to the wells in the incubator at the concentration specified. A careful analysis of the medium weight indicated that ethanol evaporates from the medium exponentially, with half-time of about 7 h (Fig. 1A). We therefore replaced the missing ethanol every 12 h, to reach the nominal concentration requested. Thus, the concentration stated (e.g. 0.5%, ≈80 mM) was actually seen only at the time of application of ethanol. Imaging and electrophysiological recording were conducted in the presence of added ethanol.

2.3. Calcium imaging and analysis

Imaging was conducted as detailed elsewhere (Korkotian et al., 2014; Cohen et al., 2008). Glass coverslips containing the cultures were incubated for 1 h at room temperature with the standard recording medium (in mM, 129 NaCl; 4 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 10 Glucose; 10 HEPES; pH 7.4, 320 mOsm), containing 2 μM Fluo-2 High Aff (AM) (TEFLabs, USA). Coverslips were placed thereafter on the stage of an inverted Zeiss 510 confocal microscope. Cells were illuminated with a low intensity (0.1–0.2% of nominal) 488-nm light and imaged at a rate of 5–10 frames per second using a 40× oil objective (NA = 1.3, Zoom = 1). Pinhole was adjusted to obtain 3 μm-thick optical slices. Fluorescence variations at 505–550 nm were recorded at room temperature except for a subset of experiments done at 37 °C, as specified below. Transients of $[Ca^{2+}]i$ resulting from action potential discharges could be clearly recorded at the imaging rate employed in these experiments (e.g. Fig. 1B). Regions of interest including neuronal somata were measured. Drugs were prepared from frozen stock solutions prior to use, and perfused into the imaging chamber. Only one "naive" field from each culture was used for imaging, so that the total duration of the experiment after the incubation did not exceed 1 h. The number of fields analyzed matches therefore the number of cultures used. Data were analyzed using Zeiss software for initial image acquisition and measurements of optical density in regions of interest. A homemade program written in MatLab was specified to automatically discriminate events, calculate the time points of spike initiation, peak and decay as well as measure the rates, durations and synchronization of firing among adjacent neurons. Coefficients of synchronization were calculated by dividing the number of synchronized spikes by the total number of spikes in adjacent neurons. Typically 5–7 neurons were analyzed in a single field of view.

2.4. Electrophysiology

The recording methods are detailed elsewhere (Fishbein and Segal, 2007). Briefly, hippocampal cultures at 13–14 days in vitro (DIV) were transferred to a recording chamber placed on the stage of an inverted Olympus IX70 microscope and washed with a standard recording medium. Neurons were recorded with patch pipettes containing (in mM): K-gluconate, 136; KCl, 10; NaCl, 5; HEPES, 10; ethylene glycol-bis (beta-amino ethyl ether) N,N,N′,N′-tetra-acetic acid (EGTA), 0.1; Na-GTP, 0.3; Mg-ATP, 1; phosphocreatine, 5; pH 7.2 with a resistance in the range of 5–8 MΩ. For recording of GABAergic synaptic activity, cells were voltage clamped at −40 mV, which allowed simultaneous recording of EPSCs and IPSCs. When recording spontaneous miniature excitatory postsynaptic currents (mEPSCs), 0.5 μM TTX and 10 μM bicuculline were added to the recording medium and neurons were clamped at −60 mV. Signals were amplified with MultiClamp 700B, recorded with pClamp9 (Axon Instruments, Foster City, CA) and analyzed with Minianalysis software (Synaptosoft).

2.5. Imaging of cell morphology

Cells were transfected with green fluorescent protein (GFP) at the age of 7 days in vitro, using lipofectamine 2000 (Korkotian et al., 2014). Control and 1% ethanol-treated cells were fixed with 4% paraformaldehyde at the age of 14–21 days in vitro, and individual neurons were imaged on the stage of a confocal microscope, 3D reconstructed, and analyzed morphologically in a blind procedure. Dendritic spines were classified into mushroom type, when the spine head was large (>1 μm) compared to the spine neck, and to filopodia which lacked a distinct head. This was based on previous classifications (see Korkotian and Segal, 2011; Korkotian et al., 2014).

2.6. Statistical analysis

One way ANOVA was used for evaluation of differences among 3 or more groups that vary in one parameter, followed, if the F value was significant, by Tukey's comparisons. Unpaired t-test was used for comparisons between two groups, assuming normal distribution. Routinely, experiments were conducted with 3–6 culture glass coverslips, in at least 2–4 litters, unless otherwise specified. For most of the imaging experiments (except when specified), one coverslip was considered a unit of observation, for the electrophysiological and morphological experiments, a neuron was the unit of observation. Value of $p < 0.05$ was considered a priori a significant difference between groups.

3. Results

3.1. Imaging of network activity

In the initial series of experiments we replicated and extended our previous observations on the effects of ethanol on network activity in cultured hippocampal neurons (Korkotian et al., 2013). Consecutive exposure of the cultures to concentrations of ethanol from 0.5% to 1% caused initially an increase in the rate of network bursts. Initial wash was associated with a marked increase in network bursts, followed by a return to control rates (Fig. 1C and D).