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Research Paper

Involvement of aberrant calcium signalling in herpetic neuralgia

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ABSTRACT

Alpha-herpesviruses, herpes simplex viruses (HSV) and varicella zoster virus (VZV), are pathogens of the peripheral nervous system. After primary infection, these viruses establish latency within sensory ganglia, while retaining the ability to reactivate. Reactivation of VZV results in herpes zoster, a condition characterized by skin lesions that leads to post-herpetic neuralgia. Recurrent reactivations of HSV, which cause mucocutaneous lesions, may also result in neuralgia. During reactivation of alpha-herpesviruses, satellite glial cells (SGCs), which surround neurons in sensory ganglia, become infected with the replicating virus. SGCs are known to contribute to neuropathic pain in a variety of animal pain models. Here we investigated how infection of short-term cultures of mouse trigeminal ganglia with HSV-1 affects communication between SGCs and neurons, and how this altered communication may increase neuronal excitability, thus contributing to herpetic neuralgia.

Mechanical stimulation of single neurons or SGCs resulted in intercellular calcium waves, which were larger in cultures infected with HSV-1. Two differences were observed between control and HSV-1 infected cultures that could account for this augmentation. Firstly, HSV-1 infection induced cell fusion among SGCs and neurons, which would facilitate the spread of calcium signals over farther distances. Secondly, using calcium imaging and intracellular electrical recordings, we found that neurons in the HSV-1 infected cultures exhibited augmented influx of calcium upon depolarization.

These virally induced changes may not only cause more neurons in the sensory ganglia to fire action potentials, but may also increase neurotransmitter release at the presynaptic terminals in the spinal cord. They are therefore likely to be contributing factors to herpetic neuralgia.

1. Introduction

Alpha-herpesviruses, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV), are pathogens of the peripheral nervous system. After primary infection, these viruses are able to establish latency within the sensory ganglia, while remaining capable of reactivating and causing disease episodes. Reactivation of VZV results in herpes zoster, a condition characterized by skin lesions that often leads to post-herpetic neuralgia (Johnson et al., 2010). Recurrent reactivations of the HSV viruses, which cause mucocutaneous lesions, may also result in neuralgia (Fatahzadeh and Schwartz, 2007; Kallio-Laine et al., 2008; Gonzales, 1992; Krohel et al., 1976).

Individual neuronal cell bodies in the sensory ganglia are surrounded by a satellite glial cell (SGC) sheath. Alpha-herpesviruses are normally found in sensory neurons during latency, although there is evidence that VZV can be found in SGCs during latency (Croen et al., 1988; Meier et al., 1993). However, during reactivation of alpha-herpesviruses, SGCs also become infected with the replicating virus (Athmanathan et al., 2001; Esiri and Tomlinson, 1972).

SGCs are not passive bystanders in the sensory ganglia, but actively contribute to neuronal signal processing and transmission (Hanani, 2012; Huang et al., 2013; Jasmin et al., 2010). A 20 nm gap exists between the neuronal membrane and the membrane of the surrounding SGCs (Hanani, 2005), allowing fast diffusion of signalling molecules between the two cell types. It is highly likely then that SGCs and neurons communicate via release of neuroactive substances, as well as by gap junctions (Hanani, 2005; Ledda et al., 2009; Suadicani et al., 2010; Wagner et al., 2014; Huang et al., 2013; Takeda et al., 2007; Laursen et al., 2014). Previous research has shown that under pathological conditions SGCs undergo a number of changes that may contribute to neuropathic pain. For example, in chronic pain states the SGC sheaths around neuronal cell bodies become extensively coupled by gap junctions, contributing to hyperexcitability of neurons and to neuropathic pain (Dublin and Hanani, 2007; Ohara et al., 2008; Warwick and Hanani, 2013; Huang et al., 2010; Blum et al., 2014). Other changes to SGCs during pain states include proliferation, increased synthesis of cytokines and up-regulation of the glial activation marker, glial fibrillary acidic protein (GFAP; Woodham et al., 1989; Takeda et al., 2007; Jasmin et al., 2010; Huang et al., 2013). In addition, in two models

Abbreviations: ATP, adenosine triphosphate; GFAP, glial fibrillary acidic protein; HSV, herpes simplex virus; ICW, intercellular calcium wave; LY, Lucifer yellow; PRV, pseudorabies virus; SGC, satellite glial cell; TG, trigeminal ganglia; VZV, varicella zoster virus.

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of inflammatory pain, the sensitivity of SGCs to the pain mediator ATP was augmented (Kushnir et al., 2011; Blum et al., 2014).

Previous research investigating how alpha-herpesviruses might cause herpetic neuralgia has completely ignored the possible contribution of SGCs (Mayer et al., 1986; McCarthy et al., 2009). In this study we asked whether alpha-herpes viral infection affects communication among SGCs and neurons, as aberrant signalling within the sensory ganglia is likely to cause sensory abnormalities. We used short-term mouse trigeminal ganglia (TG) cell cultures infected with HSV-1, to model reactivation of alpha-herpesviruses in vitro, where cells undergo lytic infection.

2. Materials and methods

The experiments were approved by the Institutional Animal Care and Use Committee of the Hebrew University-Hadassah Medical School.

2.1. Primary culture of trigeminal ganglia

Balb/c mice (males/females, 1:1) were killed with CO_2 and the trigeminal ganglia (TG) were removed. The ganglia were cleaned from connective tissue and blood vessels and transferred to a vial containing 1 mg of collagenase type 1A (Roche, Indianapolis, IN, USA) and 3 mg of Dispase II (Roche) in 1 ml of HEPES buffer with the following composition (in mM): HEPES 10, NaCl 140, KCl 3 and glucose 11; pH 7.4. The vial was incubated for 60 min in the dark at 37 °C. The collagenase/Dispase II solution was then replaced with minimal essential medium-alpha containing 10% foetal bovine serum, 100 units/ml penicillin G sodium salt, 0.1 μg/ml streptomycin sulphate, and 0.25 μg/ml amphotericin B. The ganglia were then triturated by repeated pipetting until complete tissue homogenization, and placed on MatTek glass-bottomed dishes (14 mm diameter; MatTek Corp., Ashland, MA, USA), which were then placed in a humidified 5% CO_2 incubator at 37 °C.

2.2. Phase microscopy

The cultures were observed using an inverted microscope (Nikon Diaphot TMD, Tokyo, Japan) equipped with phase optics and fluorescent illumination. Images were captured using a Cool snap HQ CCD camera (www.photometrics.com), and Image Pro Plus software (MediaCybernetics, Bethesda, MD, USA).

2.3. HSV-1 infection of primary TG cell cultures

The HSV 17+/pR20.5/5 strain with the β-galactosidase (β-Gal) gene under the control of the RSV promoter, and the GFP gene under the control of the CMV promoter was used. HSV-1 infection was carried out on 4 h old primary TG cell cultures, using a multiplicity of infection (MOI) of 0.1. Each cell culture plate was incubated for 1 h with 200 μl of minimal essential medium-alpha containing 10^3 plaque-forming units of the HSV-1 virus. Control cultures were incubated for 1 h with 200 μl of minimal essential medium-alpha without the virus. The cultures were then examined when they were 48 h old. We used young (48 h old) cultures, as SGCs undergo phenotypic changes after being cultured for longer than 48 h (Belzer et al., 2010).

2.4. Ca^{2+} microfluorimetry

Cell cultures were loaded with the Ca^{2+} indicator Quest Rhod-4 AM (AAT Bioquest, Sunnyvale, CA, USA), 2 μM in minimal essential medium-alpha for 40 min in a 37 °C, CO_2 humidified incubator. The cultures were superfused with Krebs solution, bubbled with 95% O_2 and 5% CO_2, containing (in mM): NaCl 120.9, KCl 5.9, $NaHCO_3$ 14.4, $MgSO_4$ 2.5, NaH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11.5; pH 7.3. The cell cultures were then placed on the stage of an upright microscope (Olympus BX51WI, Olympus Corp., Tokyo, Japan). Fluorescence was excited at 524 nm, and emitted fluorescence (at 551 nm) was increased by elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$). Images were recorded at 0.3 Hz. The fluorescence ratio F/F_0, where F_0 is the baseline, was used to describe relative changes in $[Ca^{2+}]_{in}$. Images were acquired with a cooled CCD camera (PCO, Kelheim, Germany), using Imaging Workbench 5 software (www.imagingworkbench.com).

For the study of intercellular Ca^{2+} waves, single SGCs and neurons were stimulated mechanically using blunt glass micropipettes driven by a micromanipulator. For experiments with ATP, cell cultures were superfused at 4 ml/min with Krebs solution containing ATP for 60 s. Similarly, Krebs solution containing 47 mM KCl was superfused for 30 s. For experiments using suramin (Sigma, St. Louis, MO, USA), cell cultures were incubated with suramin (500 μM) 20 min before the beginning of the experiment and suramin was present throughout the experiment.

2.5. Intracellular labelling (dye injections)

Cell cultures were bathed in Krebs solution saturated with 95% O_2 and 5% CO_2 throughout the experiment, and were placed on the stage of an upright microscope (Axioskop FS2, Zeiss, Jena, Germany), equipped with fluorescent illumination and a digital camera (Pixera penguin 600CL, Los Gatos, CA, USA). Individual cells were injected with either the fluorescent dye Lucifer yellow (Sigma), 3% in 0.5 M LiCl solution, or rhodamine B isothiocyanate-dextran (M.W. 10,000 Da, Sigma) 3% in 1 M KCl solution, from sharp glass microelectrodes connected to an electrometer (model IR 283, Neuro Data Instruments Corp., New York, NY, USA). Lucifer yellow was passed by hyperpolarizing current pulses, 100 ms in duration; 0.5–1 nA in amplitude at 5 Hz for 1–2 min. Rhodamine B isothiocyanate-dextran was passed by depolarizing current pulses, 100 ms in duration; 0.5–1 nA in amplitude at 5 Hz for 3–4 min. Dye injections were made under visual inspection to allow identification of the injected cell and any coupled cells.

2.6. Intracellular electrical recordings of neurons

Cell cultures were bathed in Krebs solution saturated with 95% O_2 and 5% CO_2 at 27–29 °C throughout the experiment and placed on the stage of an upright microscope (Axioskop). Sharp glass microelectrodes filled with 2 M KCl, of 80–150 MΩ tip resistance were used. Transmembrane currents were passed through the recording electrode using the bridge circuit of an electrometer. The threshold for firing an action potential was determined by measuring the minimal depolarizing current (100 ms duration) that elicited a spike. The input resistance was measured by passing hyperpolarizing currents (0.1 nA, 100 ms duration) and balancing the bridge. Electrophysiological data were analysed using pCLAMP9.

3. Results

3.1. HSV-1 infection of primary TG cell cultures

Mouse primary TG cell cultures were infected with the HSV-1 17+/pR20.5/5 strain, which expressed GFP under the control of the CMV promoter, allowing the identification of HSV-1 infected cells (Fig. 1A–F). In the HSV-1 infected cultures, 85% of SGCs were infected ($n = 324$, from 8 independent cell cultures) compared with 24% of neurons ($n = 29$; $p < 0.05$); suggesting that SGCs are more permissive than neurons for HSV-1 replication.

Differences in the appearance of the control and HSV-1 infected cultures were observed (Fig. 1). Although the total number of SGCs per neuron in each field of view was similar in the control and HSV-1 infected cultures (Fig. 1I), the distribution of SGCs was different. The number of SGCs adjoining each neuron was greater in HSV-1 infected cultures, compared with control cultures (Fig. 1J). The DAPI-stain was used to determine whether the cell body of a given SGC was adjacent to a neuronal