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Research paper

High doses of salicylate reduces glycinergic inhibition in the dorsal cochlear nucleus of the rat

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ABSTRACT

High doses of salicylate induce reversible tinnitus in experimental animals and humans, and is a common tinnitus model. Salicylate probably acts centrally and induces hyperactivity in specific auditory brainstem areas like the dorsal cochlear nucleus (DCN). However, little is known about the effect of high doses of salicylate in synapses and neurons of the DCN. Here we investigated the effects of salicylate on the excitability and evoked and spontaneous neurotransmission in the main neurons (fusiform, cartwheel and tuberculoventral) and synapses of the DCN using whole cell recordings in slices containing the DCN. For this, we incubate the slices for at least 1 h in solution with 1.4 mM salicylate, and recorded action potentials and evoked and spontaneous synaptic currents in fusiform, cartwheel (CW) and putative tuberculoventral (TBV) neurons. We found that incubation with salicylate did not affect the firing of fusiform and TBV neurons, but decreased the spontaneous firing of cartwheel neurons, without affecting AP threshold or complex spikes. Evoked and spontaneous glutamatergic neurotransmission on the fusiform and CW neurons cells was unaffected by salicylate and evoked glycinergic neurotransmission on fusiform neurons was also unchanged by salicylate. On the other hand spontaneous glycinergic transmission on fusiform neurons was reduced in the presence of salicylate. We conclude that high doses of salicylate produces a decreased inhibitor drive on DCN fusiform neurons by reducing the spontaneous firing of cartwheel neurons, but this effect is not able to increase the excitability of fusiform neurons. So, the mechanisms of salicylate-induced tinnitus are probably more complex than simple changes in the neuronal firing and basal synaptic transmission in the DCN.

1. Introduction

The dorsal cochlear nucleus (DCN) is an integrative structure of the cochlear nucleus complex. Its function is to perform a spectral analysis of sound, and integrate somatosensory information, in order to identify the vertical source of the sound (Young and Davis, 2002; Oertel and Young, 2004). The DCN has a laminar organization and its internal circuitry is basically composed of a primary output neuron (fusiform) and two inhibitory interneurons: cartwheel (CW) and tuberculoventral (TBV) neurons. Both connect to the fusiform neurons by a feedforward inhibitory synapse activated by glutamatergic synapses. The cartwheel neuron is excited by the parallel fibers (PF-CW), which also excites the apical dendrites of the fusiform neuron (Young and Davis, 2002; Oertel and Young, 2004). The TBV neuron, localized in the DCN's deep layer, is excited by synapses in the deep layer (DL-TBV) containing axons from the auditory nerve and stellate cells from the ventral cochlear nucleus which also contact the basal dendrites of the fusiform neuron (Young and Davis, 2002; Oertel and Young, 2004). Consequently, these feedforward inhibitory circuitries tightly control firing of DCN fusiform neurons, and alterations in this inhibitory-excitatory balance could potentially cause deregulation of the excitability control of the fusiform neuron.

Tinnitus is a subjective perception of a non-existent sound in the form of a constant noise and has several causes, the most prevalent are hearing loss and acoustic trauma (Eggermont and Roberts, 2004). Psychophysical evidence of tinnitus in experimental animals has been associated with hyperactivity of the DCN (Kaltenbach and Afman, 2000; Dehmel et al., 2012; Stolzberg et al., 2012; Li et al., 2013) and bilateral ablation of the DCN in mice prevented the development of tinnitus following acoustic trauma, without influencing already established tinnitus, demonstrating that the DCN is crucial for the development of tinnitus (Brozosky

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and Bauer, 2005; Brozosky et al., 2012). Indeed, animals with tinnitus induced by acoustic trauma exhibit increased burst firing and spike rate of DCN fusiform neurons (Zhang and Kaltenbach, 1998; Brozosky et al., 2002; Kaltenbach and Zhang, 2007; Li et al., 2013). Several drugs can induce tinnitus in humans and animals, including high doses of sodium salicylate (Stolzberg et al., 2012; Hayes et al., 2014). Because salicylate intoxication reliably induces tinnitus in a rapid and reversible fashion, both in experimental animals and in humans, it is commonly used to investigate the neuropathology of this disorder (Stolzberg et al., 2012). Several effects of salicylate on the auditory pathways were already described. For instance, it decreases motility of outer hair cells and, at very high doses, it increases the activity of the vestibulocochlear nerve (Kakehata and Santos-Sacchi, 1996; Guitton et al., 2003). Systemic administration of salicylate promoted hyperactivity in the DCN and inferior colliculus (IC) evaluated by magnetic resonance, electrophysiology and c-fos expression (Chen and Jastreboff, 1995; Wu et al., 2003; Holt et al., 2010). Because the central effects of salicylate are seen in lower doses than the necessary to increase the vestibulocochlear nerve activity (200 mg/kg vs 400 mg/kg; Müller et al., 2003; Kumagai, 1992; Chen and Jastreboff, 1995; Lobarinas et al., 2006), it is postulated that salicylate acts on central structures (Stolzberg et al., 2012; Hayes et al., 2014). On the other hand, studies of the mechanisms of action of salicylate on central structures, *in vitro*, were less conclusive. Perfusion of high doses of salicylate produces both an increase and a reduction in the spontaneous firing rate of action potentials in DCN neurons (Basta et al., 2008; Wei et al., 2010). In other regions such as hippocampus, auditory cortex and lateral geniculate nucleus, salicylate increases neuronal excitability by inhibiting the GABAergic transmission (Wang et al., 2006; Gong et al., 2008; Su et al., 2012). However, despite its significance for tinnitus generation, the effects of salicylate on the neurotransmission and neuronal excitability in the DCN are unknown. In this study, we investigated the effect of incubating slices containing the DCN with doses of salicylate known to be present in the cerebro-spinal fluid (CSF) of animals with salicylate-induced tinnitus, on the evoked and spontaneous excitatory and inhibitory neurotransmission at the main synapses of the DCN and on the excitability of its main neuronal types (fusiform, cartwheel and tuberculoventral). Our aim was to test the hypothesis that salicylate increases excitation of the fusiform neuron by synaptic and/or intrinsic mechanisms performing a detailed analysis of the main neurons and synapses in the DCN.

2. Materials and methods

2.1. Animals and preparation of slices

Male Wistar rats (p18-22) were obtained from the Central Animal Facility of the campus of Ribeirão Preto. All protocols were approved by the Ethics Committee on Animal Experimentation (CETEA) under the protocol 027/2012. The animals were killed by decapitation and the brains removed and cut in cold solution whose composition in mM was:NaCl (87), $NaHCO_3$ (25), KCl (2.5), NaH_2PO_4, (1.25), $CaCl_2$ (0.5), $MgCl_2$ (7), glucose (25), sucrose (75), 335 mosm/kgH_2O, pH 7.4 when bubbled with carbogenic mixture (95% O_2 and 5% CO_2). Before removing the brain from the skull, the vestibulocochlear nerve (VIII cranial nerve) was cut to prevent damage to the DCN. Coronal slices (200 μm thick) containing the 3 layers of the DCN with its basic circuit (Oertel and Young, 2004) were obtained on a vibratome (Vibratome 1000 Plus) and incubated at 35 °C for 45 min and subsequently at room temperature in recording solution (artificial cerebrospinal fluid - aCSF), whose composition in mM was: NaCl (125), KCl (2.5), $NaHCO_3$ (25), NaH_2PO_4 (1.25), glucose (10), $CaCl_2$ (2), $MgCl_2$ (1), 305 mOsm/kgH_2O, pH 7.4 when bubbled with carbogenic mixture (95% O_2 and 5% CO_2). For testing the effect of salicylate, sodium salicylate (1.4 mM) was added to the aCSF and incubated with the slices after cutting and in the perfusion solution, being present throughout the whole experiment.

For electrophysiological recordings, the slices were transferred to a perfusion chamber and fixed with a nylon thread mounted in a platinum frame. Neurons were visualized on a microscope (Olympus BX51WI) equipped with IR-DIC optics and an infrared–sensitive camera (Retiga 2000R, QImaging). The slices were continuously perfused (1 ml/min) with recording aCSF (kept at 35–37 °C with an online heater (Thermoclamp, AutoMate Scientific) with or without salicylate.

2.2. Electrophysiology

Electrophysiological recordings were obtained in whole-cell patch-clamp using an EPC-10 amplifier (HEKA Elektronik) in voltage-clamp mode to measure synaptic currents and in current-clamp mode, for measuring excitability and electrophysiological identification of neuronal types. For this, borosilicate recording electrodes (BF-150-86-10, Sutter Instruments) were pulled with a horizontal puller (P87-Sutter instruments) and back filled with internal solution containing (in mM): potassium gluconate (130), EGTA (0.01), HEPES (10), KCl (20), ATP-Mg (2), GTP-Na (0.2), phosphocreatine-Na (5); 305–310 mosm/kgH_2O, pH 7.3 adjusted with KOH, resulting in a tip resistance of 3–6 MΩ.

Fusiform, cartwheel and tuberculoventral neurons were identified by their location in the slice and their action potential pattern as further described. Voltage-current (VI) relationships were performed at resting membrane potential by injecting 1000 ms current steps from −300 to 250/400 pA with 50 pA increments. Glutamatergic excitatory post-synaptic currents (EPSCs) and glycinergic inhibitory post-synaptic currents (IPSCs) were evoked using a bipolar Ag/AgCl wire electrode inside a theta-glass micropipette, filled with aCSF, and recorded at a holding potential of −80 mV. All EPSCs were recorded in the presence of 1 μM of strychnine (STR) and 20 μM of picrotoxin (PTX). Parallel fibers EPSCs were generated by placing the electrode in the molecular layer and recorded both in fusiform (PF-FUS) and cartwheel (PF-CW) neurons. EPSCs from the auditory pathway (the auditory nerve and stellate cells from the VCN) were evoked by placing the electrode in the myelinated deep layer (DL) and moving it until we evoked an EPSC, and recorded both in fusiform (DL-FUS) and tuberculoventral neurons (DL-TBV neurons). All EPSCs were evoked using the voltage that produced a maximum response. EPSCs were inhibited by 10 μM of 6,7-dinitroquinoxaline-2,3-dione (DNQX) (not shown) confirming their glutamatergic nature. Glycinergic IPSCs were evoked in the presence of 20 μM PTX and 10 μM of DNQX and recorded in fusiform neurons. PTX did not affect the amplitude of the sIPSCs (control: 29.6 ± 7 pA; PTX: 28.4 ± 5 pA; $p = 0.55$, paired t-test; $n = 5$). The theta electrode was placed in the molecular or deep layer and moved until we evoked an IPSC. IPSCs expressed an all-or-nothing fashion suggesting they were originated from a single neuron (cartwheel or tubeculoventral). The glycinergic nature of the IPSCs was confirmed by their complete inhibition by STR (data not shown). A measured liquid junction potential of 10 mV was off-line corrected. Acceptable series resistance was below 30 MΩ.

EPSCs and IPSCs were stimulated every 30 s by delivering a single or a double voltage pulse (100 ms interval) using a Grass SD9 pulse stimulator. At least 30 currents were evoked for analysis. Spontaneous glutamatergic EPSCs and glycinergic IPSCs were isolated pharmacologically and recorded for 5 min at −80 mV.