Salicylate enhances expression and function of NMDA receptors in cochlear spiral ganglion neurons

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Abstract Objective To study the effect of salicylate on the expression and function of NMDA receptors in spiral ganglion neurons (SGNs).

Methods The mRNA of NR1 subunit of NMDA receptor in modiolus tissues were detected by Real time fluorescence quantitative PCR (FQ–PCR). NMDA receptor whole–cell currents were recorded using patch clamp in acute isolated SGNs.

Results Compared with the control group, salicylate significantly increased the mRNA level of NR1 subunit in SGNs. NMDA of concentrations ranging from 0.1 mM to 10 mM evoked no current in SGNs. NMDA (0.1 mM and 0.5 mM) applied with salicylate (5 mM), however, induced inward currents (212.6±15.2 pA, n=5; 607.9±44.3 pA, n=5) in a dose–dependent manner, which could be inhibited by APV. Salicylate alone did not produce any current in SGNs.

Conclusion Salicylate increases the expression of NMDA receptors and facilitates the currents mediated by NMDA receptors in SGNs.

Key words Salicylate; NMDA receptor; spiral ganglion neurons; NR1 subunit

Introduction

Aspirin has been widely used for purposes of anti–inflammation, relieving pains, and treating cardiovascular diseases for more than 100 years. Salicylate is the major metabolic product and active component of aspirin. Overdose of salicylate can result in many side effects, such as gastrointestinal bleeding, liver disorders, allergic reactions, reversible tinnitus and hearing loss of 30 dB HL. Most of studies suggest that the ototoxicity induced by salicylate is related with impairment of cochlea, e.g. salicylate reduces blood flow in the cochlea 1, elevates the intracellular concentration of calcium in cochlea 2, or changes the morphology and electromotility of hair cells 3,4. However, the spontaneous activities and the threshold of action potential in auditory afferent pathway are increased, while the amplitude of action potential is reduced after salicylate is applied. Salicylate does not change the microphonic potential 5. These electrophysiology results can hardly contributed to cochlear impairment. Therefore, we hypothesize that salicylate induced ototoxicity may be concerned with auditory neurons dysfunction.

Spiral ganglion neurons (SGNs) are the primary auditory neurons, providing the afferent innervation of the hair cells and connection with the auditory center. Studying SGNs may provide insight into mechanisms of auditory physiological and pathophysiological processes. Our previous studies have proved that all the functional NMDA receptor subunits are expressed in SGNs 6,7. This implies that the glutamate receptors, e.g. NMDA receptors, may be involved in auditory neurotransmission. Thus, in the present study, we tested the effect of salicylate on mRNA level of NR1 subunit of NMDA receptor by using Real time fluorescence quantitative PCR (FQ–PCR) and the whole–cell currents of NMDA receptors in SGNs using whole–cell patch clamp technique. The results showed that salicylate apparently increases the expression of NMDA receptors and then facilitates the NMDA receptor currents in SGNs.
Methods

Primary culture SGN

SGN culture was conducted as described previously\(^7,8\). In brief, cochlear spiral ganglion tissues were dissected from postnatal 3–5 days Wistar rats. Following cervical decapitation the cochlea was quickly removed under a stereomicroscope and placed in cold D-hanks solution at 4°C. The cochlear capsule was then opened. The modiolus containing spiral ganglion was isolated. The modiolus was divided as two parts. One was collected in sterilized EP tubes for FQ-PCR. The other was minced into small pieces and transferred into the D-hanks solution containing 0.25% trypsin at 37 °C for 10 min. The trypsin was then inactivated by adding DMEM/F12 (HyClone, USA) containing 10% fetal calf serum (FCS) (Gybicol, USA) to the solution. Following centrifugation at 800 (r/min) for 8 min and the removal of supernatant, the pellet was re-suspended and gently triturated. Dissociated tissue was then plated onto poly-D-lysine-coated 35 mm culture dishes and cultured with DMEM/F12 containing 10% FCS for 6 to 8 hours in a humidified CO2 (5%) incubator at 37 °C before performing electrophysiological experiments. The cells that were large, oval and bipolar were selected for patch clamp recordings. All animal experiments were conducted following the guidelines of, and approved by the Animal Care and Use Committee at Guangxi Medical University.

FQ-PCR assay

The methods used for mRNA extraction and DNA synthesis were similar to that described previously\(^6,7\). In brief, Trizol reagent (Invitrogen, USA) was used for extracting total RNA from modiolar tissues. Synthesis of NR1 cDNA was determined by Reverse Transcriptase Kit. 4% of the synthesized products were used for estimating the expression of NR1. Total RNA was reverse–transcribed into cDNA according to manufacturer’s protocol by (FQ–PCR) assay based on SYBR Green I dye detection. The assay was conducted using the ABI7500 system in a total volume of 20 μl reaction mixture following the protocol of manufacture, using the 2.5x RealMasterMix (TIANGEN) and 0.2 μM of each of the following primers: rat NR1 forward: 5’ –AGGAGTGGGAGGATGAT–3’, reverse: 5’ –ACTTGAAGGCTTGAGAATGAC–3’; rat glyceraldehyde–3–phosphate dehydrogenase (GAPDH) forward: 5’ –GGCACAGTCAAGGCTGAGAATG–3’, reverse: 5’ –ATGGGTTGAAGACGCCAGTA–3’. Base on the preliminary experiments result, the annealing temperature was set at 58 °C and the amplification cycles were set at 40 cycles. The melting curve and subsequent sequencing of the FQ–PCR products were measured to examine the specificity of the primers. Distilled water was used for negative control for all of the reactions. NR1 mRNAs was normalized on the basis of its GAPDH mRNA content in each sample. To achieve comparison between samples, the threshold cycle (Ct) was calculated for determining the relative expression of the gene interested. The Ct of the GAPDH gene was from the Ct of the target gene yielding the ΔCt in each DIV point, and subtracted for yielding the Ct in each group. The Ct was obtained by subtracting from other DIV groups, and the ΔCt of the control (3DIV) group was entered into the equation 2\(^{-\Delta \Delta Ct}\) and calculated for the exponential amplification of PCR. The results were presented as mean ± SD from 3 independent experiments with 3 replicates for each data point.

Electrophysiology

Whole–cell patch clamp recording methods in SGNs have been described in detail previously\(^8\). In brief, SGN cultures were placed in a recording chamber on an inverted microscope (IX 51, Olympus, Japan). The morphology of SGNs was monitored. No significantly change was found on the size or shape of SGNs by either the bath solutions or experimental manipulations. SGNs were bathed in a standard extracellular solution composed of (in mM): NaCl (150), KCl (3), CaCl2 (4), Glycine (0.01), HEPES (10), glucose (5). The pH of the solutions was adjusted to 7.4. Recording electrodes were pulled from borosilicate glass capillaries using a P–97 puller, and filled with intracellular solution containing (in mM): KCl (145), CaCl2 (0.1), HEPES (5), EGTA (11). The pH of the solution was adjusted to 7.2. Standard extracellular solution containing TTX (0.3 μM) was applied, after TTX–sensitive voltage gated sodium current was identified in SGNs. DC resistances of recording electrodes were 2 – 5 M. To detect voltage gated sodium currents, SGNs were held at −70 mV and depolarized from −70 mV to −10 mV for 25 ms. Drugs used in this study were dissolved in the extracellular
solution and perfused for 1 s via a multi-barrel perfusion system (ALA perfusion system, USA). To test whether changes in expression of NMDA receptors is related to up-regulation on NMDA receptor function, NMDA receptor currents were recorded with EPC–10 amplifier (HEKA, Germany) in the whole-cell configuration. Recordings were conducted under the voltage–clamp condition at a holding potential of −70 mV except where indicated. Online data acquisition and off-line analysis were performed using the Pulse8.0 and Pulse-fit8.61 software (HEKA, Germany). Results were shown as mean ± SD. Statistical significance between the means of different concentrations of NMDA applied with sodium salicylate was determined with Student t test.

**Results**

**Influence of salicylate on expression of NMDA receptors in SGNs**

Our previous studies have demonstrated that NMDA receptor subunits, including NR1, NR2A–2D and NR3, are all detected in SGNs6,7. The results of FQ–PCR showed that upon exposure to salicylate (5 mM), ΔCt value of NR1 mRNA in SGNs was gradually reduced (Table 1). Compared with the control group, which was bathed with culture medium, the relative change of ΔCt was significantly increased after salicylate was applied for 4 hours, and maximized at 16 hours (Figure 1). The ΔCt of NR1 mRNA in control group did not show any change.

<table>
<thead>
<tr>
<th>Time</th>
<th>Control (ΔCt)</th>
<th>Salicylate (ΔCt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2h</td>
<td>9.05±0.26</td>
<td>8.76±0.35</td>
</tr>
<tr>
<td>4h</td>
<td>9.03±0.24</td>
<td>8.15±0.48*</td>
</tr>
<tr>
<td>8h</td>
<td>9.09±0.20</td>
<td>8.06±0.26*</td>
</tr>
<tr>
<td>16h</td>
<td>8.99±0.15</td>
<td>7.84±0.26*</td>
</tr>
</tbody>
</table>

Table 1 The effect of sodium salicylate on ΔCt of NMDA receptor NR1 subunit in SGN. *: P<0.01.

**Regulation of NMDA receptor whole–cell currents in SGNs by salicylate**

(Figure 2) shows that the inward current induced by changing the holding potential to −10 mV was blocked by TTX (3 μM), indicating that the inward current is TTX–sensitive voltage gated sodium channel currents, and that the cell is a SGN. Because more than 95% of SGNs are classified as type I, most of recorded SGNs in this work were very likely type I. To test whether the NMDA receptor was activated by NMDA in SGNs, NMDA was applied at 0.1, 0.5, 1, 5 and 10 mM with a multiple perfusion system. (Figure 3) shows that various concentrations of NMDA application did not induce any current response. However, there was an inward current (212.6±15.2pA, n=5) induced by NMDA (0.1 mM) when applied in combination with sodium salicylate (5mM) (Figure 4A). Co–application of 0.5 mM NMDA and sodium salicylate (5 mM) induced apparently larger inward current (607.9 ± 44.3pA, n=5) than that induced by 0.1 mM NMDA plus sodium salicylate in SGNs (P<0.05, Student t-test) (Figure 4B). The inward current could be blocked by NMDA receptor selective antagonist APV (≥90%), an NMDA receptor selective antagonist, indicating that it was mediated by the NMDA receptor. Sodium salicylate (5mM) alone did not evoke any current in SGNs as well (Figure 4C).

**Figure 2** Examples of recorded inward current traces before (Control), during and after (Wash) TTX application.

**Figure 3** Representative whole–cell recording in SGNs after application of various concentrations of NMDA (0.1mM–10mM).
Figure 4  The effect of sodium salicylate on NMDA receptor mediated currents in SGNs. A: Whole-cell currents induced by application of NMDA 0.1mM with 5mM sodium salicylate, NMDA 0.1mM with 5mM sodium salicylate plus 0.2 mM APV, NMDA 0.1mM with 5mM sodium salicylate. B: Currents from SGNs after application of NMDA 0.5mM with 5mM sodium salicylate, NMDA 0.5mM with 5mM sodium salicylate plus 0.2 mM APV, NMDA 0.5mM with 5mM sodium salicylate. C: Currents recorded from SGNs after applied 5mM sodium salicylate.

Discussion

NMDA receptor is a type of ionotropic glutamate receptor. The NMDA receptor subunits have been identified: NR1 subunit, a family of four NR2 subunits (NR2A, NR2B, NR2C, NR2D), and two NR3 subunits. The functional NMDA receptor is formed as a heteromeric between NR1 subunit and NR2A and/or NR2B subunit. It has been proved that the receptor channel pore, which is permeable with $K^+$, $Na^+$ and $Ca^{2+}$, is composed of NR1 and NR2 subunits. Instead of forming functional NMDA receptor alone, NR3 subunits can only co-assemble with NR1 and NR2 subunits into receptor complex.[9,10] It is considered that NR1 is obligatory for a functional NMDA receptor[12].

Kuriyama and colleagues find that NR1 mRNA is selectively expressed in SGNs.[13] Recently NR2 and NR3 subunits are also found to be detected in SGNs by FQ–PCR.[6,7]

Although NMDA receptors are present in SGNs, their functions in auditory transmission remain unclear. It is considered that NMDA receptors do not involve in fast postsynaptic transmission, since cochlear perfusion of APV does not produce influence on the cochlear potential[14]. Chen et al indicate that AMPA receptor trafficking in SGNs plays a predominant role in auditory sensitive regulation. NMDA receptors regulates the number of AMPA receptors on the surface of SGNs to participate in modulating synaptic efficiency[15].

Aspirin, a widely used ant–inflammation medicine, can induce ototoxic side effects, including reversible tinnitus and hearing loss. Peng et al find that aspirin selectively potentiates NMDA receptor whole-cell currents, while having little effect on AMPA receptors and Kainate receptors in SGNs[16]. Salicylate, the major active component of aspirin, also increases NMDA receptor responses in cochlear slices[17]. These findings imply that NMDA receptors, not the non-NMDA receptors, may be the major target of ototoxicity induced by salicylate in SGNs. Our present data show that compared with control group, the mRNA of NMDA receptor NR1 subunit in SGNs is dramatically increased after salicylate treatment, suggesting that the expression of NMDA receptors in SGNs is up-regulated by salicylate. This is similar as the findings reported previously. Exposure to salicylate potentiates the expression of NR2A in auditory cortex, or NR2B in cochlea and midbrain[18,19].

Nakagawa et al and Ruel et al have reported respectively that there is no detectable NMDA receptor current during NMDA application in acute isolated SGNs. In the present study, various concentrations of NMDA (0.1–10mM) did not induce any current in SGNs. It is likely that NMDA receptors in SGNs cannot be activated by NMDA directly. Conversely, Lin and colleagues find that 0.5 mM NMDA induces a small inward current (25±4pA) in the SGNs isolated from postnatal mice, which is blocked by APV[16]. Thus, whether NMDA receptors in SGNs can be activated by NMDA is still disputed. The electrophysiological property of ion channel usually is determined by the conductance, the gating and the number of channels expressed on the membrane. One possibility is that the number of NMDA receptors expressed on the membrane of SGNs may remain at a low level, causing a NMDA receptor mediated current that is too small to be detected in normal situation. In excitotoxic injury induced by surgery, the gating properties, the trafficking or the expression of NMDA receptors in SGNs can be altered[14]. This
may be the reason detectable NMDA receptor responses are recorded by NMDA administration. More detailed studies dealing with NMDA receptor activation in SGNs are needed.

Though NMDA alone did not induce detectable response in SGNs, there was obvious inward current evoked by NMDA (0.1 and 0.5 mM) when applied with salicylate. This current induced by NMDA was blocked by APV, which means it was mediated by NMDA receptors. Salicylate alone did not produce any response indicating that salicylate facilitates NMDA receptor mediated currents in SGNs. This is in agreement with the results of Ruel et al, who found that NMDA administrated with salicylate increased NMDA receptor currents in cochlear slice. The mechanisms by which salicylate enables cochlear NMDA receptor currents may be associated with arachidonic acid increased in cochlea after exposed to salicylate, which has been proved to potentiate NMDA receptors by increasing the channel open probability. Considering all these results, we hypothesis that the mechanisms of salicylate induced ototoxicity include increased number of NMDA receptors expressed in SGNs, especially the expression of NR1 subunit, and increased the arachidonic acid levels in the cochlea. NMDA receptors may be overly activated, and a large amount of Ca$_{2+}$ flows into SGNs triggering the downstream enzyme to go on to damage the structure of SGNs, including components of the cytoskeleton, membrane, and DNA.

It is known that the function of central auditory neurons is modulated via different pathways by salicylate. In the hippocampus, salicylate has no effect on glutamate evoked currents, but significantly reduces the GABA$_{A}$ receptor currents in a concentration–dependent manner. In the inferior colliculus, the content of GABA and the number of GABA$_{A}$ receptor binding site are decreased by increasing glutamic acid decarboxylase (GAD) after application of salicylate. Furthermore, it can inhibit either the GABAergic sIPSCs directly, or the 5-HT–induced enhancement of GABAergic sIPSCs in inferior colliculus or cochlear nucleus. All the results above demonstrate that salicylate enhances the excitation of neurons in brain via reduction of GABA$_{A}$ receptor mediated inhibitory neurotransmission. Besides, salicylate also inhibits the voltage gated ion channels, e.g. potassium, sodium and calcium channels in inferior colliculus and auditory cortex. These are considered one of the mechanisms of salicylate induced tinnitus. Therefore, the neural and synaptic function is regulated by salicylate through multiple pathways.

It is known that tinnitus induced by salicylate can be attenuated through perilymphatic perfusion of NMDA receptor antagonist (MK801 or 7–chlorokynurenate) in rats. Patients with unilateral deafness associated with tinnitus also experience a temporary relief after constant local application of gacyclidine into the cochlea (40–63 hours). Thus, characterizing the regulation of NMDA receptors by salicylate will be important for the development of therapeutics for treating ototoxicity induced by ototoxic agent in the auditory system.

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Reference


