

Effect of hydrogen peroxide on electrical coupling between identified *Lymnaea* neurones

Alexander V. Sidorov

Department of Physiology, Belarusian State University,

Nezhaleznasty Av., 4, Minsk, 220030,

The Republic of Belarus

Fax number: + 375 17 209-58-08

Phone number: + 375 17 209-58-06

E-mail: sidorov@bsu.by

Abstract

The pair of giant reciprocally coupled neurons VD1 and RPaD2 within the CNS of the fresh water pond snail *Lymnaea stagnalis* was used to analyse the effect of hydrogen peroxide on gap junction connection. Electrical activity of VD1/RPaD2 was recorded with intracellular microelectrodes in order to analyse gap junction signalling. Hydrogen peroxide application (1×10^{-4} M) results in a rapid, 1.3 fold, increase of VD1/RPaD2 spiking frequency within 30 sec after application. This was accompanied by a slight reduction in action potential amplitude. In addition, H_2O_2 induced a significant reduction in the steady state bi-directional coupling ratio between the neurons. The maximal reduction in the coupling ratio, 1.8–1.9 fold, was measured 3 min after H_2O_2 application. However, the network input resistance did not undergo a detectable change. The voltage-gated Ca^{2+} channel blocker, nifedipine (1×10^{-4} M), abolished the effect of H_2O_2 on the coupling ratio and firing frequency. All the effects of H_2O_2 were reversible i.e. washing the preparation with standard physiological saline restored the properties of the neuronal coupling to the pre-treatment value. These data are consistent with a dynamic modulation of the gap junction properties by H_2O_2 between these two neurons.

Key words: Reactive oxygen species; Gap junction; Identified neurons; Synapse; Mollusc

Introduction

Electrical coupling between cells is a common phenomenon through the animal kingdom both in neuronal and non-neuronal tissues (Bennet 1997). Its presence may contribute in different ways to the integrative properties of neuronal networks such as spike synchrony and rhythm generation (Egelhaaf and Benjamin 1983; Getting 1989; Jefferys 1995). Gap junctions form one class of electrical synapses. They are organized by two cell-to-cell hemi-channels permeable to small molecules (Bennet et al. 1991). The conductance of gap junctions is sensitive to various modulators including intracellular calcium, temperature, pH and dopamine (Rose and Loewenstein 1976; Loewenstein 1981; Johnston and Ramon 1981; Heitler and Edwards 1998; Spray et al. 1981; McMahon and Knapp 1989).

Reactive oxygen species (ROS) – the superoxide anion, O_2^- , hydrogen peroxide, H_2O_2 and the hydroxyl radical, OH^\cdot are initially produced as a side product of electron transport during oxidative phosphorylation in mitochondria (Turrens 2003). In the past two decades a number of references have reported the involvement of ROS both in intra- and intercellular signalling (Finkel 1998; Pellmar 1995). Among them H_2O_2 is of particular interest due to its stability in the extracellular space and the ability to pass through the cell membrane (Murrant and Reid 2001; Weiss 1986). Several groups have reported a neuromodulatory role for hydrogen peroxide with respect to chemical transmission for various types of synapses (Colton et al. 1986; Giniatullin and Giniatullin 2003; Katsuki et al. 1997). However, the effects of ROS on electrical transmission within neuronal networks has not been discussed.

The pair of giant peptidergic neurons VD1 and RPaD2 in the central nervous system (CNS) of the fresh water pond snail *Lymnaea stagnalis* was originally described in the early 1970s (Benjamin and Ings 1972). These cells are coupled by a low-resistance reciprocal electrotonic junction and can serve as a model for studying the properties of electrical synapses (Benjamin and Pilkington 1986). Thus the aim of the present work is to analyse the effect of hydrogen peroxide on the electrical

properties of an identified gap junction connection in the CNS of this model invertebrate, *L. stagnalis*.

Materials and Methods

Specimens of *L. stagnalis* (L.) were collected locally, kept for up to 4 weeks in tap water at 20–22 °C and fed on lettuce. All experiments were conducted on snails weighing 2–4g. The animals were anaesthetized in 0.2 M MgCl₂ (Elofsson et al. 1993). Isolated brains of molluscs were bathed in normal physiological saline (at 20°C) which consisted of (mM): NaCl 44.0, KCl 1.7, CaCl₂ 4.0, MgCl₂ 1.5, HEPES 10.0. The pH was adjusted to 7.5 with 0.1 M NaOH. Zero Ca²⁺/ high Mg²⁺ (CaCl₂ was removed and the concentration of MgCl₂ was increased 6 times in comparison with normal saline) physiological saline was also used in some experiments. Protease E type XIV (Sigma) was used to soften the perineurium (1 mg/ml for 5 min at 20°C). Individual neurones were impaled with glass microelectrodes filled with 2.5M KCl; electrode resistance 10–20 MΩ. Neurones (VD1 and RPaD2) were identified according to their location, size, colour and electrophysiological characteristics (see the map in Winlow and Benjamin 1976). Electrophysiological signals were amplified, displayed on an oscilloscope and recorded by conventional means (pen recorder). The temperature was maintained at 20 ± 0.2 °C using a laboratory made thermostat based on a Peltier assembly. Electrophysiological measurements were made with two, single-electrode current/voltage clamp amplifiers. The current clamp technique was used for gap junction analysis (Wildering et al. 1991). Under current clamp condition both cells were alternately injected with a constant hyperpolarizing current (1 nA, 2 s). The membranes of the injected neuron (i.e. prejunctional cell) as well as the non-injected neuron (i.e. postjunctional cell) were hyperpolarized. The coupling ratio between cells 1 and 2 (CR₁₂ and CR₂₁) was measured as $CR_{12} = \Delta V_{RPaD2} / \Delta V_{VD1}$ and $CR_{21} = \Delta V_{VD1} / \Delta V_{RPaD2}$, when current was injected into VD1 and RPaD2 respectively. The input resistance of this bidirectional network was measured as $R_{12} = \Delta V_{VD1} / I$ and $R_{21} = \Delta V_{RPaD2} / I$ current injected in VD1 and RPaD2 respectively. Hydrogen peroxide

and nifedipine were added into the experimental chamber and all concentrations indicated in the text are the final concentrations.

Results

In the CNS preparations both VD1 and RPaD2 were spontaneously active. Due to the prominent electrical coupling both neurons fire their spikes simultaneously (1:1). Hydrogen peroxide application (1×10^{-4} M) results in significant changes in the electrical properties of these neurons (fig. 1). This is expressed in a rapid, 1.3 fold, increase of VD1/RPaD2 spiking frequency within 30 sec after application, followed by its gradual decline. This response was accompanied by a slight reduction of action potential amplitude both in VD1 and RPaD2. In this case the membrane potential value of RPaD2 remains invariable during the course of experiment ($F = 1.23$; $P = 0.32$, $n = 7$, one-way ANOVA test). Meanwhile, VD1 was depolarized ($F = 5.06$; $P = 0.0025$, $n = 10$, one-way ANOVA test) to a maximal value of 10.1 ± 2.5 mV between 1 to 2 min after H_2O_2 application. Washing the preparation with normal physiological saline significantly restored the electrical properties of VD1/RPaD2 to their initial values. At lower concentrations, i.e. 1×10^{-5} – 1×10^{-6} M, H_2O_2 had no significant effects on spiking frequency, amplitude or membrane potential.

Hydrogen peroxide application (1×10^{-4} M) also had effects on the electrical properties of this simple bidirectional neuronal network (fig. 2, 3). Hydrogen peroxide induced a significant reduction in steady state coupling ratio in both directions. The maximal response was measured at the interval from 3 to 4 min after application: 1.9 and 1.8 fold reduction for CR_{12} and CR_{21} respectively compared to their pre-application value. Interestingly, but total network input resistance, R_{12} and R_{21} both, almost did not undergo significant changes after H_2O_2 application. This can be explained by very low contribution of gap junctions resistance to the resting resistance of the neuron, as compared to the overall membrane resistance of the cells. As previously was point out, hydrogen peroxide at 1×10^{-5} – 1×10^{-6} M was noneffective and had no significant effects on electrical

be blocked by different free radical scavenging agents. It is hypothesised that hydrogen peroxide is responsible for phosphorylation of Cx43 and extracellular signal-regulated protein kinase 1/2 which are critical for the regulation of this type of communication (Kim et al. 2009). Similar effects were reported for protein kinase C (PKC) gamma, a unique isoform of PKC that is found in neuronal cells. H₂O₂ (100 microM for 20 min) caused oxidation of the C1 domain, activation of the PKC-gamma, phosphorylation of the Cx43 gap junction proteins on Ser-368 and inhibition of gap junctions in rabbit lens epithelial cells and a mouse neuroblastoma cell line (Lin and Takemoto 2005). On the other hand, in L2, a Rat Lung Epithelial cell line, which express both Cx32 and Cx43, and Marshall cells, a fibroblastoid rat mammary tumor cell line, which express Cx43, ROS (H₂O₂, 1 mM, 3 min after treatment) depolarized cell membranes and opened connexin hemichannels (Ramachandran et al. 2007). Moreover a 10 min exposure to 100 microM H₂O₂ increased Cx43 gap junction communication in cultured rat astrocytes (Rouach et al. 2004).

It is well known that the conductance of gap junctions may be changed by either altering the number of connexons or by gating connexons already present. Changes in connexion turnover often takes place in time scales longer than an hour (Bennet et al. 1991; Saez et al. 2003). In our experiments the coupling between VD1 and RPaD2 neurons is dynamically modulated by hydrogen peroxide. The effects were observed within several minutes. These rapid (minutes) and reversible changes in junctional conductance support the idea of second messenger involvement in gating processes. Previously we reported the modulation of VD1/RPaD2 electrical coupling by cyclic nucleotide analogues (Sidorov et al. 1999). The decrease of the coupling ratio could also be related to the effect of hydrogen peroxide on the voltage gating of junctional conductance (Trexler et al. 1996). A slight depolarization of the VD1 cell was observed during the experiment but this occurred earlier (1–2 min after H₂O₂ application) than the effect on the coupling ratio (later than 3 min after H₂O₂ application). Action potential modulation of Cx40 gap junction conductance in mouse neuroblastoma cells has also been reported (Lin and Veenstra 2004). In our experiments VD1/RPaD2 spike frequency increases with the same time course as the effect described for

membrane potential, i. e. there was no significant correlation between the effect on action potential frequency and electrical coupling. Taken together, these observations indicate there may be a role for second messengers in control of gap junction permeation in the VD1/RPaD2 network.

The fact that hydrogen peroxide effects at 1×10^{-4} M were abolished by nifedipine supports the idea that the control of VD1/RPaD2 gap junction permeability by ROS involved some Ca^{2+} -dependent mechanism. The sensitivity to nifedipine points to a possible role for classical voltage-gated Ca^{2+} channels, the major route for Ca^{2+} entry into neuronal cells (Catterall 2011). Previously it was reported that gap junction communication is highly sensitive to the cytoplasmic concentration of free calcium ions (Rose and Loewenstein 1976) and intracellular Ca^{2+} homeostasis components are modified by ROS (Camello-Almaraz et al. 2006). Meanwhile, at higher millimolar concentration the effects of hydrogen peroxide can be realized due to direct oxidation of membrane proteins, for example connexins.

The data presented here demonstrate that in the CNS of *Lymnaea stagnalis* (i) hydrogen peroxide exerts a modulatory effect on the electrically coupled neurons VD1 and RPaD2; (ii) it can provide *dynamic modulation* of gap junctional conductance in normal physiological conditions within neuronal networks. These observations prompt the suggestion that ROS can be potent regulators of cell-to-cell communication processes in brain and that this may be mediated via Ca^{2+} dependent mechanisms.

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coupling. Washing the preparation with standard physiological saline did elevated CR_{12} and CR_{21} meanings to a level not significantly different from pre-application value.

Hydrogen peroxide application (1×10^{-4} M) performed in zero Ca^{2+} /high Mg^{2+} physiological saline ($n = 3$) leads to similar changes in electrical activity of the neurons under study in comparison compared to those observed in normal physiological saline. Thus there was a slight increase in spike frequency in the first 30 sec after H_2O_2 application. However, a significant effect on the value of the resting membrane potential value and action potential amplitude was not observed for both neurons. The VD1/RPaD2 network input resistance and coupling ratio also did not alter after hydrogen peroxide application (1×10^{-4} M). Addition of high H_2O_2 concentrations (1×10^{-3} M) decreased the coupling ratio by 1.5-2 times (CR_{12} and CR_{21} decreased from $33.3 \pm 1.9 \%$ and $31.0 \pm 2.0 \%$ to $15.0 \pm 1.2 \%$ and $20.7 \pm 2.1 \%$, respectively; 3–4-min exposure to 1×10^{-3} M H_2O_2). This effect was similar to the effect, which was induced by 1×10^{-4} M H_2O_2 in normal physiological saline. Exposure to 1×10^{-3} M H_2O_2 did not induce significant changes in the values of bidirectional network input resistance.

Application of 1×10^{-4} M nifedipine, a voltage-gated Ca^{2+} channel blocker, almost totally blocked the effects of hydrogen peroxide (1×10^{-4} M) on VD1/RPaD2 coupling (see table 1 for details). Nifedipine itself at this concentration had no significant effects on the coupling ratio or on the network input resistance within VD1/RPaD2 neurons. A small, 1.4 fold, but significant decrease of CR_{12} vs control was observed even after nifedipine treatment when of H_2O_2 was applied at 1×10^{-3} M.

Discussion

The possible role of ROS, and particularly of hydrogen peroxide in the modification of electrical coupling has been shown for several non-neuronal tissues both in pathological and non-pathological conditions. For instance, H_2O_2 (500 microM) induced inhibition of gap-junction intercellular communication through connexin-43 (Cx43) channels in rat liver epithelial cells and this effect can

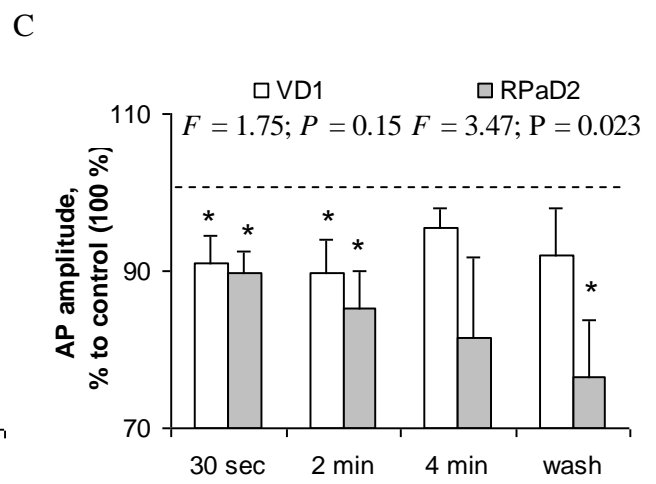
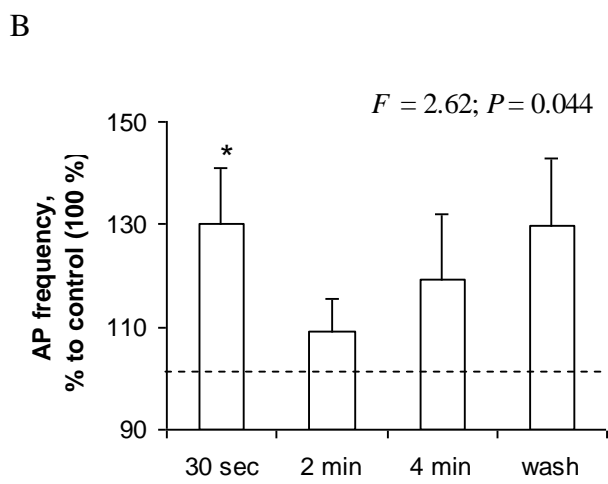
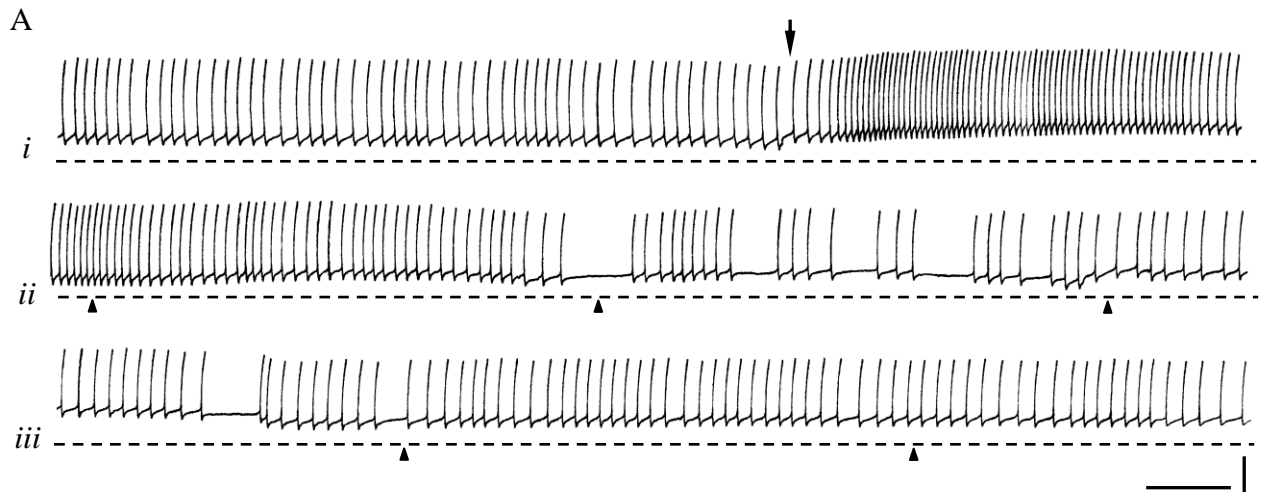
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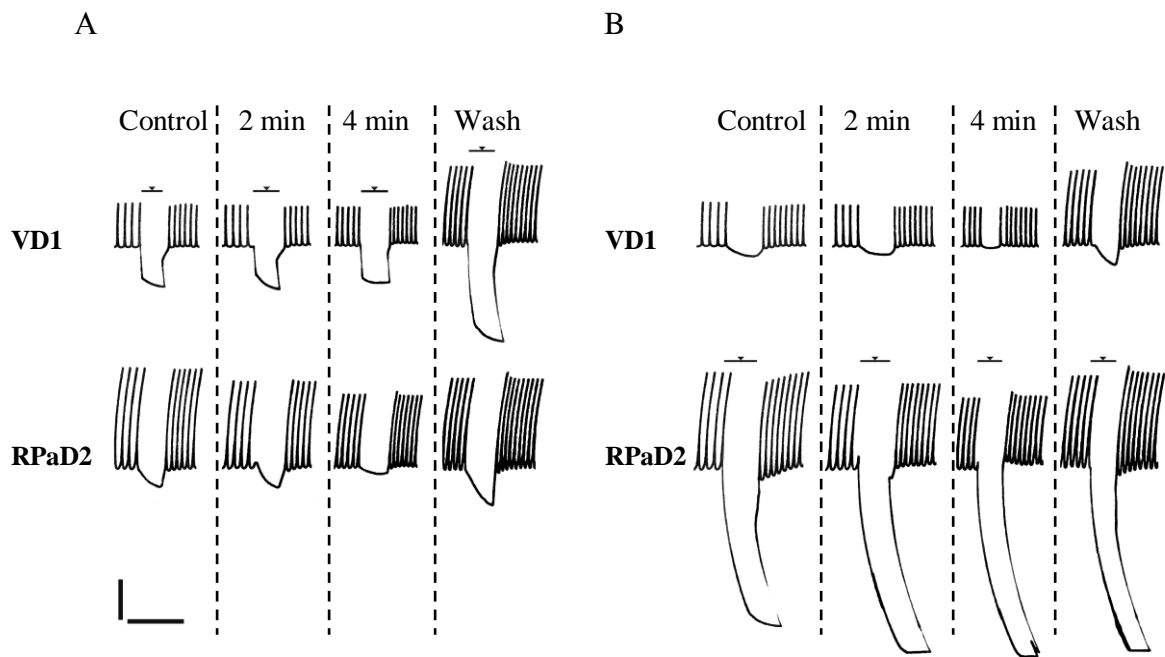
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Table 1. Effect of hydrogen peroxide on electrical coupling within VD1/RPaD2 network after nifedipine treatment.

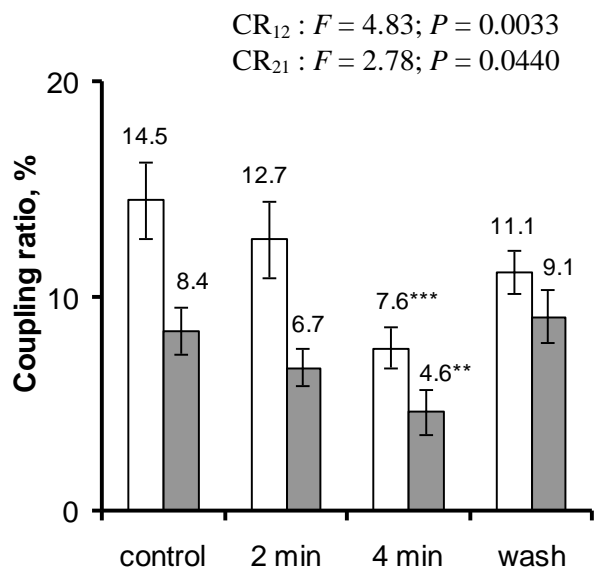
VD1/RPaD2 gap junction properties	Experimental series						
	Control	Nifedipine (1 x 10 ⁻⁴ M)	H ₂ O ₂ (1 x 10 ⁻⁴ M), after nifedipine treatment		H ₂ O ₂ (1 x 10 ⁻³ M), after nifedipine treatment		Wash
			2 min	4 min	2 min	4 min	
Coupling ratio (CR ₁₂), % of coupling	45.7±4.2 (n = 23)	39.3±2.4 (n = 32)	45.7±3.2 (n = 20)	39.4±2.8 (n = 8)	40.7±4.1 (n = 18)	32.5±5.3* (n = 10)	37.5±4.1 (n = 23)
Coupling ratio (CR ₂₁), % of coupling	28.0±4.1 (n = 22)	29.3±3.0 (n = 32)	33.7±5.4 (n = 18)	21.6±4.2 (n = 8)	35.2±4.3 (n = 19)	25.1±3.1 (n = 10)	31.4±3.1 (n = 22)
VD1/RPaD2 gap junction properties	Experimental series						
	Control	Nifedipine (1 x 10 ⁻⁴ M)	H ₂ O ₂ (1 x 10 ⁻⁴ M), after nifedipine treatment		H ₂ O ₂ (1 x 10 ⁻³ M), after nifedipine treatment		Wash
			2 min	4 min	2 min	4 min	
Bidirectional network input resistance (R ₁₂), MΩ	24.0±2.5 (n = 23)	21.3±2.8 (n = 32)	36.2±4.7** (n = 20)	20.3±4.0 (n = 8)	26.1±4.3 (n = 18)	22.2±4.3 (n = 10)	28.6±5.1 (n = 23)
Bidirectional network input resistance (R ₂₁), MΩ	35.1±3.4 (n = 22)	28.8±3.3 (n = 32)	41.9±3.9 (n = 18)	30.8±6.0 (n = 8)	32.5±5.0 (n = 19)	29.2±5.2 (n = 10)	24.6±4.7 (n = 22)

Average values (coupling ratio and network input resistance) are presented for the control conditions, 1 min before H₂O₂ application (control), after nifedipine application (nifedipine), at time intervals from 1 to 2 min (2 min), and from 3 to 4 min (4 min) after H₂O₂ application, and after washing in normal physiological saline (wash). Data present mean value ± SEM. * – significant (P = 0.045), ** – significant (P = 0.020) relative to control value (one-way ANOVA test). Number of CNS preparations = 7, number of observations (n) presented individually for each measurement.





A



B

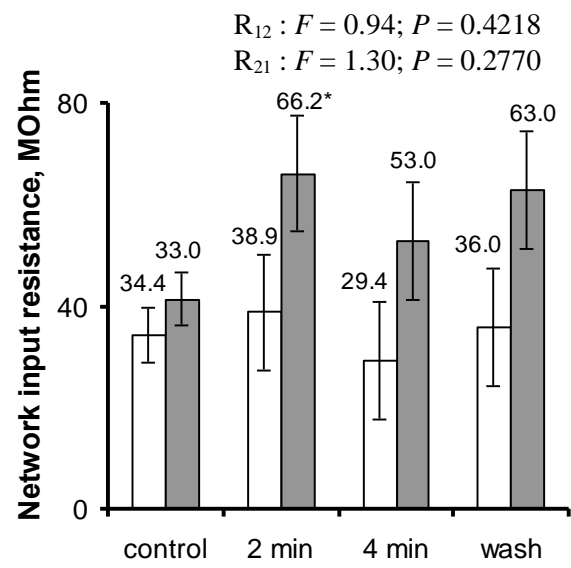


Fig. 1. Effect of hydrogen peroxide on the spontaneous electrical activity of VD1/RPaD2 neurons.

A – Continuous traces of VD1 electrical activity (i–iii) are given. Hydrogen peroxide application (1×10^{-4} M) is marked by the arrow. The dashed line is used for visualization of membrane potential alterations. Triangles – time marks (1 min) after H_2O_2 application. Calibration bars: 10 sec (time), 50 mV (voltage).

B – Action potential (AP) generation frequency. C – Action potential amplitude.

Average values presented for the control conditions, 1 min before H_2O_2 application (control), at time intervals from 0 to 30 sec (30 sec), and from 1 to 2 min (2 min), 3 to 4 min (4 min) after H_2O_2 application, and after washing in normal physiological saline (wash). Number of neurons studied: VD1: $n = 12$, RPaD2: $n = 7$. Data present mean value \pm SEM. One-way ANOVA test (for F and P meanings). * – significant ($P < 0.05$), Students t-test.

Fig. 2. Effect of hydrogen peroxide on electrical coupling between VD1/RPaD2 neurons.

Hyperpolarizing current pulses (marked by the bars with triangles) applied to VD1 (A) and RPaD2 (B). Membrane potential in VD1 – top trace, in RPaD2 – bottom trace.

Control – control conditions (before H_2O_2 application), 2 min – value for the interval from 1 to 2 min after H_2O_2 application, 4 min – value for the interval from 3 to 4 min after H_2O_2 application, wash – after washing.

Calibration bars: time – 5 sec, voltage – for VD1: 20 mV (control, 2 min, 4 min), 10 mV (wash), for RPaD2 – 10 mV.

Fig. 3. Effect of hydrogen peroxide on coupling ratio (A) and network input resistance (B) within VD1/RPaD2 network under current clamp conditions.

Average values presented for the control conditions, 1 min before H₂O₂ application (control), at time intervals from 1 to 2 min (2 min), and from 3 to 4 min (4 min) after H₂O₂ application, and after washing in normal physiological saline (wash). Forward direction (CR₁₂, R₁₂) – light histogram bars, inverse direction (CR₂₁, R₂₁) – dark histogram bars. Data present mean value (numbers above the columns) ± SEM. Number of CNS preparations: n = 7, number of observations: n = 30 for each characteristic. One-way ANOVA test meanings presented (F and P values), * – significant (P < 0.05), ** – significant (P < 0.01), *** – significant (P < 0.001), relative to the control value, Students t-test.