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Modulation of 4-aminopyridine-induced neuronal activity and local pO_2 in rat hippocampal slices by changing the flow rate of the superfusion medium

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The brain slice preparation is the most frequently used tool for testing of pharmacological agents on the neuronal excitability. However, in the absence of blood circulation in vitro, the tissue oxygenation strongly depends on the experimental conditions. It is well established that both hypoxia as well as hyperoxia can modulate the neuronal network activity. Thereby changes in tissue oxygen level during experiment may affect the final result. In the present study we investigated the effect of oxygenation on seizure susceptibility in the hippocampal slice preparation using 4-aminopyridine (4-AP) model of ictogenesis in immature rats. We found that changing the medium perfusion rate in the range of 1-5 ml/min greatly affects the tissue oxygenation, amplitude and frequency of 4-AP-induced synchronous neuronal activity. The decrease in the flow rate as well as substitution of the oxygen in the extracellular medium with nitrogen causes a strong reduction of 4-AP-induced synchronous neuronal discharges. Our results demonstrate a significant linear correlation between the power of 4-AP-induced neuronal activity and the oxygen level in slice tissue. Also we demonstrated that the presence of medium flow is a necessary condition to support the constant level of the slice oxygenation. These data suggest that the oxygen supply of the brain slice strongly depends on experimental protocol and could modulate in vitro neuronal network excitability which should be taken into consideration when planning epilepsy-related studies.

Key words: *brain slices; synchronous neuronal activity; oxygen; local field potential.*

INTRODUCTION

Acutely isolated brain slices have been introduced for the study of seizure-like activity several decades ago [1] and remained a widely used research object [2,3]. The tissue oxygenation and energy supply in the brain slice preparation differ from that in the intact brain, where vascular system supplies oxygen and nutrients to nervous cells to support its normal activity [4,5]. Due to the absence of the functional vascular system in the brain slices, oxygen delivery to the neuronal tissue is limited by its diffusion from the extracellular environment and may depend on experimental conditions [6].

For testing of pharmacological agents on neuronal excitability in the brain slice preparation researchers most frequently use a submerged chamber. In this type of chamber the oxygen tension strongly depends on oxygen concentration at the "medium-slice" border and decreases with the distance from the slice surface. Recent studies reported that the ability of neuronal network to generate of the pharmacologically-induced gamma oscillation (30-100 Hz) in submerged slices depends on the flow rate [7]. High-resolution measuring of a local partial pressure oxygen (pO_2) *in vitro* during gamma oscillations shows more then 2-fold increase in the oxygen consumption rate compared with a spontaneous asynchronous neuronal network activity [8]. Slow diffusion of the oxygen into tissue may lead to the strong decreasing the oxygen level in the slice core. Lower oxygenation resulted in the decrease of the local field potentials and spontaneous

network activity as well as in the significant modulation of short-term synaptic plasticity [8]. On the other hand, hypoxia and anoxia conditions may play a substantial role in the induction of synchronic network oscillation and may cause "hypoxia-induced" epileptiform seizures in the newborns that occur in various conditions [9,10]. Unlike adults, in newborns these seizures are often refractory to medical treatment and may lead to epileptogenesys. The mechanisms underlying the increased seizure susceptibility of the immature brain to hypoxia are not clear.

The aim of the present study was to investigate the effect of perfusion rate and the level of oxygen in extracellular environment on pharmacologically induced synchronous neuronal activity in submerged brain slices dissected from immature rats. To induce the epileptiform activity we used the specific antagonist of voltage-gated potassium channel, 4-aminopyridine (4-AP), which was shown to reliably induce prolong seizures-like activity in brain slices and frequently used as a tool for testing potential therapies of pharmacoresistant seizures in acute slice preparation.

METHODS

Hippocampal slices preparation.
All procedures used in this study were approved by the Animal Care Committee of Bogomoletz Institute of Physiology. Briefly, Wistar rat pups at postnatal day 10-12 were anesthetized with inhalation of isoflurane and decapitated. The brain was removed and placed in the ice-cold oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl 126; KCl 3.5; $CaCl_2$ 2.0; $MgCl_2$ 1.3; $NaHCO_3$ 25; NaH_2PO_4 1.2; and glucose 11 (pH 7.4). Sagittal hippocampal slices (500 μm) were cut using a vibroslicer (NVSL, World Precision Instrument) and kept before recordings for at least an hour at room temperature (22-24°C) in a submerged chamber containing oxygenated ACSF.

Electrophysiological recordings.
Extracellular field potential recordings were performed using patch pipettes made from borosilicate glass capillaries and filled with ACSF. Pipette resistance was ranged from 1-3 MΩ. Recordings were made from CA1 pyramidal cell layer of hippocampus. Signals were amplified using AC differential *amplifier* (A-M Systems, Carlsborg, WA) (bandpass 0.1 Hz -1kHz; x100), digitized (10 kHz) online with an analogue-to-digital converter (NI PCI-6221, National instruments, Austin, TX) and stored on a computer using WinWCP 4.05 (Strathclyde Electrophysiology Software, University of Strathclyde, Glasgow, UK).

Oxygen Measurements.
Oxygen was measured 100-150 μm above the slice surface or within slice tissue using Clark-style microelectrodes fabricated in our laboratory from glass capillary tube (outer diameter 1.5 mm), platinum wire (~10 μm diameter) and home-made polarographic amplifier. Before each experiment the oxygen sensor was calibrated in the recording chamber. The oxygen sensor was polarized by square pulses (at -800 mV, duration 1 s, and frequency 0.1 Hz). Changes in the pO_2 was determined as the difference in the current amplitude recorded in the oxygen-saturated ACSF (bubbled with 95% O_2 /5% CO_2), oxygen-free ACSF (bubbled with 95% N_2 /5% CO_2) and oxygen-free 1 mM Na_2SO_3 dissolved in non-bubbled ACSF at 2 ml/min medium flow rate. During experiments, ACSF bubbling with 95% N_2/5% CO_2 was used to substitute the oxygen from medium. Changes in the medium flow rate did not affect the oxygen level in the recording chamber (Fig.1A). In all experiments the oxygen-current amplitude was normalized to the current amplitude measured in oxygen-saturated ACSF at 2 ml/min flow rate.

The oxygen electrode was positioned in hippocampal CA1 region at the proximity to field potential recording electrode (Fig. 1B). Fig 1C demonstrated that the level of oxygen is gradually decreased with the immersion of

oxygen electrode into slice tissue. At 2 ml/min medium flow the relative oxygen level at 100 μm depth was 21±5%, which corresponds to normoxic conditions. So all recordings of the electrical activity and pO_2 was performed at this depth.

Statistical analyses

Off-line analysis of the recordings was performed using Clampfit 9.5 (Molecular Devices, Sunnyvale, CA) and Origin 8.0 (Microcal corp, Northampton, MA) software. Data are presented as mean ± SE. Datasets were compared by investigator "blinded" to experimental conditions. Statistical analysis was performed using paired Student's t-test. Pearson's product–moment correlation coefficient was used to estimate dependence between two variables.

Power spectrum of synchronous network oscillations were calculated by fast Fourier transformation (Bartlett window, FFT bin size 0.1 Hz) for 60 s data segments each at three perfusion rate: a) 2 ml/min (control condition), b) 5 ml/min and c) 1 ml/min. In experiment with a constant flow rate and non-perfusion chamber, 60 s data segments were analyzed at 10 min (control condition) and 30 min after 4-AP application. For comparison of power spectrum at different perfusion rate, the sum of the power of the bins from 1 to 30 Hz was calculated and normalized to the control conditions.

RESULTS

Epileptiform activity was induced by application of 4-AP and both field potentials and slice oxygen levels were measured in pyramidal CA1 region of hippocampus in slices prepared from P10-12 rats. 4-AP (50 μM) applied at 2 ml/min perfusion rate evoked synchronous neuronal

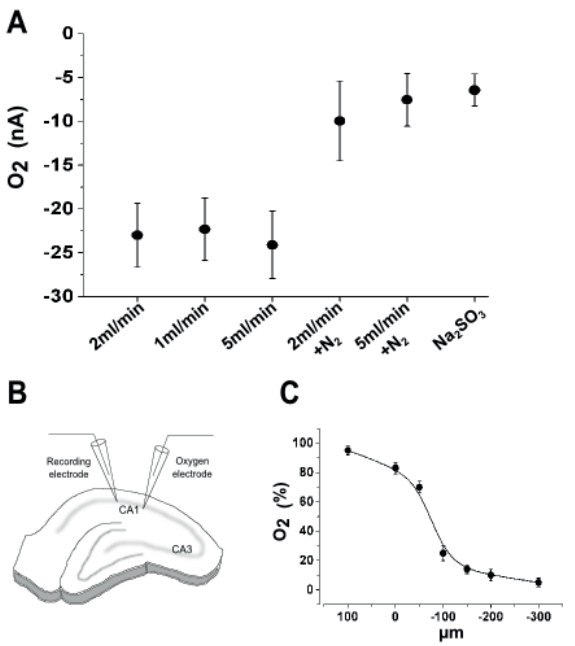

Fig.1. Dependence of the oxygen level on the flow rate and slice depth. (A) Measurement of oxygen level in the submerged-type slice chamber at different perfusion rates in ACSF saturated with 95% O_2 /5% CO_2, ACSF saturated with 95% N_2 /5% CO_2 or 1 mM Na_2SO_3 dissolved in 0.9 % NaCl. (B) Schematic illustration showing position of recording and oxygen electrodes on the hippocampal slice. (C) Oxygen depth profile in the hippocampal slice tissue recorded at 2 ml/min perfusion rate. Values are ± SEM

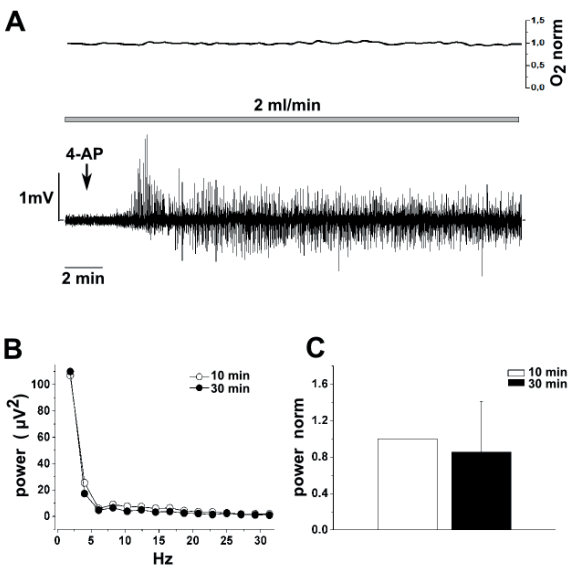

Fig.2. Synchronous network oscillations induced by 4-AP and oxygen level in hippocampal CA1 pyramidal region of slices maintained in a submerged-type chamber. (A) Original recording of 4-AP-induced synchronous neuronal activity (bottom) and oxygen level (top) at 2 ml/min perfusion rate. (B) Power of synchronous oscillations presented on A at the frequency band between 0 and 30 Hz (60 sec segment) at 10 min and 30 min after application of 4-AP. (C) Cumulative histogram of power spectrum of synchronous activity under constant perfusion rate at 10 min and 30 min after application of 4-AP. Values are ± SEM

discharges within a few minutes (Fig.2A, bottom trace). The level of slice oxygenation did not change during the recording (Fig. 2A, top trace). The stability of synchronous oscillations was estimated using power spectrum analysis (Fig. 2B). Cumulative data from 6 recordings demonstrates that synchronous neuronal activity became stable within a few minutes after application of 4-AP: the power of synchronous oscillations at the frequency band between 0 and 30 Hz at 10 min and 30 min after application of 4-AP was not significantly different (Fig. 2C, $n = 6$, $P = 0.08$, paired Student's t-test).

Figure 3 demonstrates the level of slice oxygenation and 4-AP-induced synchronous activity in slice perfused at different flow rates. At 1, 2 and 5 ml/min medium flow rate, the average power of synchronous discharges was 97.1 ±17.2 μV, 104.8 ±23.7 μV and 156.1 ±28.1 μV, respectively (n = 10). There was a significant difference in the power of synchronous neuronal activity recorded at 2 ml/min and 5 ml/min perfusion rate (Fig. 3C, $P = 0.009$, paired Student's t-test). The decrease of the perfusion rate from 2 ml/min to 1 ml/min did not result in the change of the power of synchronous discharges (Fig. 3C, $P = 0.37$, paired Student's t-test). Figure 3D demonstrates the level of slice oxygenation at the different rates of medium perfusion. We observed an insignificant decrease of the slice oxygen level at 1ml/min flow rate (67 ± 19%, $n = 10$, $P = 0.054$, paired Student's t-test) and the increase of the level of slice oxygenation to 226 ± 35% at 5 ml/min ($n = 10$, $P = 0.012$, paired Student's t-test) compared to the slice oxygen level recorded at 2 ml/min flow rate.

To test whether the presence of a medium flow is a necessary condition to support the constant level of the slice oxygenation, the experiment presented in Fig. 2A was performed in the modified submerged type chamber,

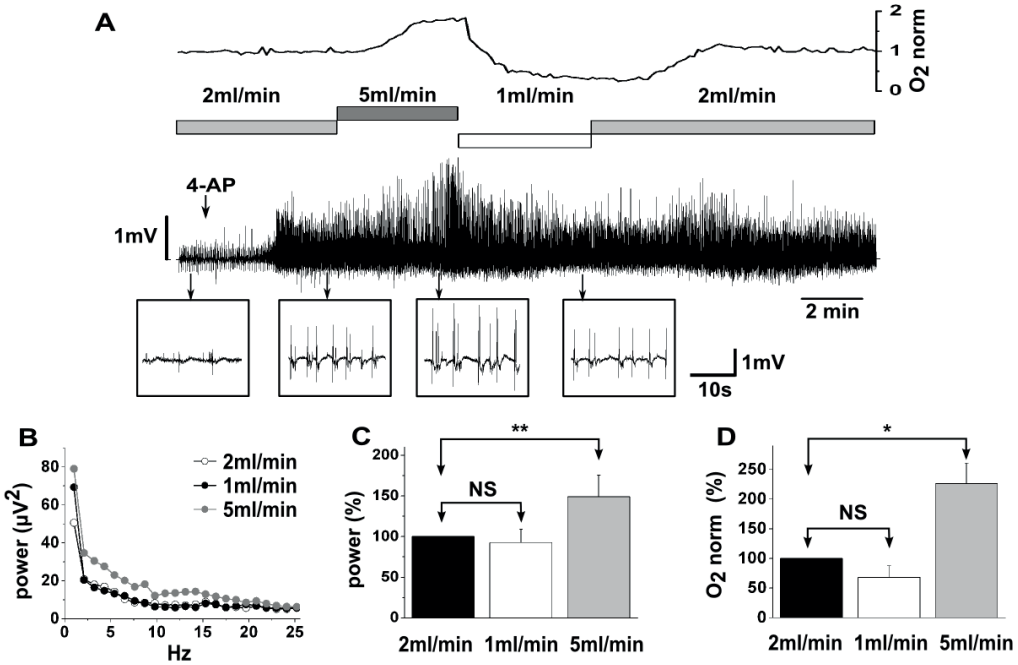

Fig. 3. Synchronous network activity and oxygen level recorded in pyramidal CA1 region strongly depend on the perfusion rate. (A) Raw recording of 4-AP-induced synchronous network oscillations (bottom) and oxygen level (top) at 1, 2 and 5 ml/min perfusion rate (indicated by horizontal bars). Inset boxes indicate the field potential recordings on expanded scales. (B) Power spectrum of field potentials presented on A at the frequency band between 0 and 30 Hz at different flow rates. The average power of synchronous activity (C) and level of slice oxygenation (D) at different flow rates. Values are ± SEM. Statistical comparisons were performed using Student's t-test * $P < 0.05$

which provides a saturation of the medium by oxygen without a flow superfusion (Fig. 4A). Modified submerged-type slice chamber has no perfusion ports for extracellular solution. Oxygen saturated gas mixture comes directly to the chamber through holes in the tube located around the perimeter of the chamber. Slices were placed on the nylon mesh which provides a better slice oxygenation. The slices were dipped in oxygen-saturated ACSF containing 50 μM 4-AP. The level of oxygen in these experiments was measured in the medium at a distance of 100-150 μm above the slice surface and at 100 μm slice depth. While the level of oxygenation in the medium during recording was constant, the oxygen level in the slice was gradually decreased (n = 5, P = 0.03, paired Student's t-test, Fig. 4B, C). The power of synchronous network oscillations induced by application of 4-AP also decreased during recording time (Fig. 4D). An average power of synchronous neuronal discharges was significantly different at 10 min and 30 min after application of 4-AP (Fig. 4E, n = 5, P = 0.0006, paired Student's t-test).

Next we examine the effect of 4-AP on neuronal network activity in anoxic conditions. The simple hypoxia (anoxia) in hippocampal slices was achieved by substitution of oxygen-saturated (95% O_2/5% CO_2) for oxygen-free (95% N_2/5% CO_2) gas mixture. Oxygen deprivation led to the rapid decrease in the 4-AP-induced neuronal activity recorded at 2 ml/min flow rate. In the condition of oxygen deprivation, the increase of the perfusion rate to 5 ml/min resulted in a further drop of the power of synchronous neuronal activity (Fig. 5A). An average power of synchronous neuronal activity was significantly lower at 2 ml/min in oxygen-zero ACSF (Fig. 5B, dark-gray, n = 6, P = 0.003, paired Student's t-test) compare to the recordings in oxygen-saturated conditions. Changing the medium flow rate from 2 ml/min to 5 ml/min in oxygen-zero ACSF led to further decrease in the power of synchronous activity (Fig. 5B, light-gray, n = 5,

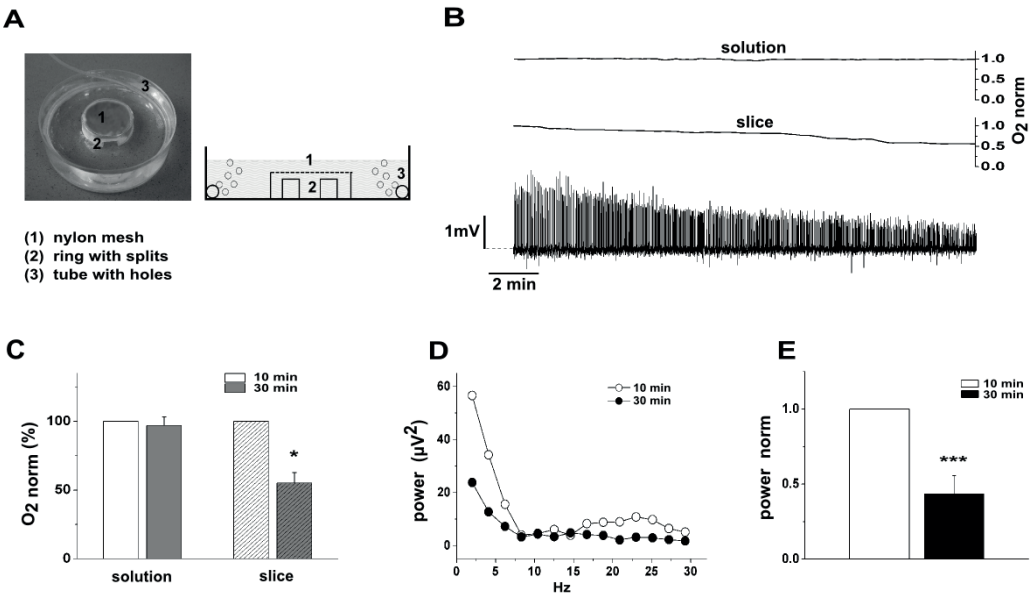

Fig.4. 4-AP-induced synchronous neuronal activity and oxygen level recorded in hippocampal CA1 pyramidal cell layer in a non-perfusion slice chamber. (A) Modified submerged-type slice chamber. (B) 4-AP-induced synchronous network oscillations (bottom trace) and oxygen level measured in external medium at 100-150 μm above the slice surface (top trace "solution") and at 100 μm slice depth (middle trace "slice"). (C) Cumulative histogram of the oxygen level in the medium and in slice recorded at 10 min and 30 min after 4-AP application. (D) Power spectrum for the recording presented on B at the frequency band between 0 and 30 Hz at 10 min and 30 min (1 min segment) after 4-AP application. (E) Average power of induced synchronous activity at 10 and 30 min after application of 4-AP

P = 0.0045, two-sample Student's *t*-test). Fig. 5C demonstrates the summary of in-slice changes of pO2 in this set of experiments. Oxygen deprivation at 2 ml/min flow rate led to a significant reduction of the slice oxygen level (*n* = 6, *P* = 0.0008, paired Student's *t*-test). The change of the medium flow rate from 2 ml/min to 5 ml/min in oxygen-free medium led to further reduction of slice oxygenation level (*n* = 5, *P* = 0.0006, two-sample Student's *t*-test). Fig. 5D shows a strong linear correlation between the power of 4-AP-induced synchronous neuronal activity and measured oxygen level in slices (R = 0.91, Pearson's correlation).

DISCUSSION

Our results demonstrated that 4-AP-induced seizure-like neuronal activity and the oxygen level in submerged hippocampal slices strongly depend on the perfusion rate.

It was reported previously, that the ability of neuronal network to generate of the pharmacologically-induced high frequency oscillation in submerged slices depends on extracellular medium flow rate [8]. The seizure-like synchronous neuronal activity is an energy-intensive process and requires significant energetic substrate consumption. Decrease of the oxygen level during electrophysiological recording of high frequency oscillation in brain slice may reflect both activity-dependent oxygen consumption and limitation of oxygen diffusion into the slice tissue [11,12]. Studies of neuronal function *in vivo* performed under normobaric conditions suggest that the ambient atmosphere contains 21 % oxygen at a pressure of 750 Torr or 1 atmosphere absolute (1 ATA; "normobaric normoxia"). Under this condition pO_2 in brain tissue measured by a polarographic

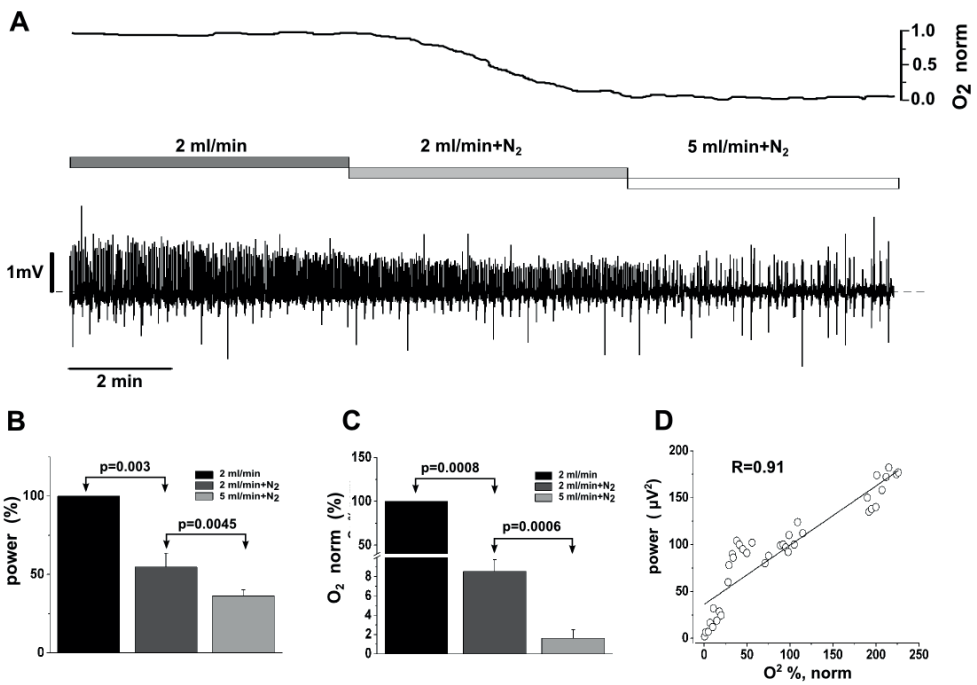

Fig. 5. Synchronous neuronal activity and oxygen level recorded in hippocampal CA1 pyramidal cell layer in the conditions of oxygen deprivation. (A) Field potential recording of the 4-AP-induced synchronous activity (bottom) and oxygen level (top) at 2 ml/min flow rate in oxygen-saturated medium (95% O_2/5% CO_2) and at different flow rates in oxygen-free medium (95% N_2/5% CO_2) (flow rate indicated by horizontal bars). The average power spectrum of synchronous neuronal discharges (B) and oxygen level (C) at 2 ml/min flow rate in oxygen-saturated ACSF (black) and at 2 ml/min (dark grey) and 5 ml/min (grey) in oxygen-free ACSF. (D) Relationship between the power of synchronous neuronal activity and slice oxygen level

or magnetic resonance method was 10-60 Torr that is markedly different from that used for brain slices (100-700 Torr) [7,12]. Actually the pO_2 at normobaric pressure in the superfusion medium in the brain slice experiments is hyperoxic compared to the intact brain and mimics pO_2 in neuronal tissue *in vivo* during "hyperbaric hyperoxia" administered pressure (> 1 ATA) of 100 % O_2. In our recordings the oxygen depth profile reflects the dynamic of oxygen diffusion into slice tissue (Fig. 1C) and demonstrates a gradual decrease of the oxygen concentration, when oxygen electrode moved away from upper surface. Our data shows that normobaric hyperoxygenated ASCF (95% O2/5% CO_2 at normal atmospheric pressure) at flow rate 2 ml/min provides constant normoxic conditions at 100-150 μm depth of slice. These results are close to previous studies investigating the oxygen profile in the brain slice tissue under various perfusion rate and oxygen concentrations [7,13]. In contrast, in the conditions of the absence of medium flow in the recording chamber we observed progressive reduction in the pO_2 as well as decrease in the power of 4-AP-induced synchronous neuronal activity.

In the intact brain the blood circulation system delivers oxygen to neurons via sophisticated mechanism of the vascular-neuronal interaction. Neurons, glial cells and vessels create the "neurovascular units" which regulate a neuronal activity and neuronal plasticity. In the acute brain slice preparation oxygen has to diffuse into the tissue due to the absence of active transport. The balance between the diffusion flow of oxygen into the tissue and amount of oxygen consumed by cells is required to keep a steady state conditions [14, 15].

At a laminar medium flow, the concentration border layer with a low diffusion gas gradient is formed on the slice surface. Based on the kinetic equation:

$$r = kC, \qquad (I)$$

where r is a reaction rate, k - reaction rate constant and C is chemical concentration, the rate of oxygen transport r_O from medium to cells in brain slices can be expressed as:

$$r_O = (K_d K_{DBL} / K_d + 1/K_{DBL})C \qquad (II)$$

where K_d is a diffusion coefficient into tissue and K_{DBL} is a diffusion coefficient across border layer. From the equation II, the total oxygen diffusion coefficient (K_{total}) expresses as:

$$1/K_{total} = 1/K_d + 1/K_{DBL} \qquad (III)$$

In our experiments K_d was constant. Also the presence of oxygen-saturated medium in the chamber without a flow superfusion could not support a constant level of in-tissue oxygenation (Fig. 4B). We suggest that the maintaining of the optimal medium flow is required to provide the balance between oxygen delivery and oxygen consumption during a high frequency neuronal activity.

It was shown previously, that neuronal activity and synaptic transmission are suppressed following transient cerebral ischemia [16]. Other studies report, that transient ischemia lead to the synaptic potentiation and increases of the spontaneous firing rate recorded in CA1 region of anesthetized animals [17]. The global hypoxia induces seizures in P10-12 rats in vivo. Also the increased seizure susceptibility was observed in hippocampal slices obtained from the hypoxia-treated rats [10]. In our experiments we did not see seizure-like activity under hypoxia conditions. Moreover, reducing PO_2 in the tissue linearly decreases the intensity of epileptic discharges (Fig. 5D). The mechanism of hypoxia-induced epileptiform discharges is not clear, but likely involves changes in the transmembrane ion conductivity, the release of the reactive oxygen species and disturbance of the mitochondrial redox state. We suggest that under our experimental conditions a low oxygen level does not affect these mechanisms.

By contrast, it was shown that hyperoxia leads to the activation of population spikes and synchronous interictal discharges in CA1 hippocampal region [18]. Oxygen-induced plasticity may play a substantial role in the pathogenesis of CNS. For example, the oxygen toxicity-induced seizures in rats occur during the inhalation of 3 ATA of 100% O_2 for several

hours [19]. Also increasing the neural tissue pO_2 from the normoxic range (10 – 45 Torr) to 240 Torr or more caused tonic-clonic seizures in cats [20]. Our data support these findings and show that the increase in the perfusion rate resulted in the rapid rise of PO_2 and increase in the synchronous neuronal activity.

In summary, we suggest that severity of the pharmacologically-induced seizure-like activity in *vitro* is determined by the tissue oxygen level that is a complex function which depends on experimental conditions, including ambient pO_2, perfusion flow rate and position of the recording electrode in the slice tissue. Based on our kinetic model, future electrophysiological experiment on submerged brain slices addressing the synchronous epileptiform activity should take into account a slow rate of oxygen equilibration in tissue for correct interpretation of results.

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ЗМІНА ШВИДКОСТІ СУПЕРФУЗІЇ ПРИЗВОДИТЬ ДО МОДУЛЯЦІЇ ІНДУКОВАНОЇ 4-АМІНОПІРИДИНОМ НЕЙРОННОЇ АКТИВНОСТІ ТА ЛОКАЛЬНОГО ВМІСТУ КИСНЮ В ЗРІЗАХ ГІПОКАМПА ЩУРІВ

Із використанням 4-амінопіридинової (4-АР) моделі ікотогенезу у незрілих щурів ми вивчали вплив оксигенації на здатність зрізів гіпокампа генерувати епілептиформну активність. Виявлено, що зміна швидкості перфузії в діапазоні 1-5 мл/хв призводила до модифікації амплітуди і частоти 4-АР-індукованої синхронної активності нейронів, а також до змін вмісту кисню у зовнішньо клітинному середовищі. Зниження швидкості потоку, а також видалення кисню з позаклітинного розчину з використанням азоту, призводило до швидкої редукції вмісту кисню в нервовій тканині і викликало значне зниження потужності синхронних нейронних розрядів. Показано позитивну лінійну кореляцію між потужністю 4-АР-індукованої епілептиформної активності нейронів і вмістом кисню. Також виявлено, що наявність перфузії є необхідною умовою для підтримки постійного рівня оксигенації зрізу. Отримані результати дають змогу стверджувати, що спосіб постачання кисню до тканин зрізів мозку залежить від експериментального протоколу і може модулювати in vitro нейронну мережеву збудливість, що слід брати до уваги при плануванні експериментів, пов'язаних з дослідженням епілепсії.

Ключові слова: зрізи мозку; синхронна нейронна активність; кисень; локальний польовий потенціал.

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ИЗМЕНЕНИЕ СКОРОСТИ СУПЕРФУЗИИ ПРИВОДИТ К МОДУЛЯЦИИ ИНДУЦИРОВАННОЙ 4-АМИНОПИРИДИНОМ НЕЙРОННОЙ АКТИВНОСТИ И ЛОКАЛЬНОГО УРОВНЯ КИСЛОРОДА В СРЕЗАХ ГИППОКАМПА КРЫС

Мы изучали влияние оксигенации на способность срезов гиппокампа генерировать эпилептиформную активность с использованием 4-аминопиридиновой (4-АР) модели иктогенеза у молодых крыс. Показано, что изменение скорости перфузии в диапазоне 1-5 мл/мин приводило к модуляции амплитуды и частоты 4-АР-индуцированной синхронной активности нейронов, а также к изменениям уровня кислорода. Снижение скорости суперфузии, также как и вытеснение кислорода из внеклеточного раствора азотом, приводило к быстрому снижению уровня кислорода в нейронной ткани и вызывало значительное снижение мощности 4-АР-индуцированных синхронных нейронных разрядов. Показано положительную линейную корреляцию между мощностью 4-АР-индуцированной активности нейронов и уровнем кислорода в ткани среза. Также продемонстрировано, что наличие суперфузии является условием для поддержания постоянного уровня оксигенации среза. Результаты данной работы позволяют предположить, что транспорт кислорода к тканям срезов мозга зависит от экспериментального протокола и может модулировать возбудимость нейронной сети in vitro, что следует учитывать при планировании экспериментов, связанных с исследованиями эпилепсии.

Ключевые слова: срезы мозга; синхронная нейронная активность; кислород; локальный полевой потенциал.

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