Maturation of neuronal excitability in hippocampal neurons of mice chronically exposed to cyclic hypoxia

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Gu, Xiang Q., and Gabriel G. Haddad. Maturation of neuronal excitability in hippocampal neurons of mice chronically exposed to cyclic hypoxia. Am J Physiol Cell Physiol 284: C1156–C1163, 2003; 10.1152/ajpcell.00432.2002.—To examine the effects of chronic cyclic hypoxia on neuronal excitability and function in mice, we exposed mice to cyclic hypoxia for 8 h daily (9 cycles/h) for 2 wk (starting at 2–3 days of age) and examined the properties of freshly dissociated hippocampal neurons obtained from slices. Compared with control (Con) hippocampal CA1 neurons, exposed neurons (CYC) had similar resting membrane potentials (Vm), action potentials (AP), and Na+ channel action potentials (AP). CYC neurons, however, had a lower rheobase than Con neurons. There was also an upregulation of the Na+ current density (333 ± 84 pA/pF, n = 18) in CYC compared with that of Con neurons (193 ± 20 pA/pF, n = 27, P < 0.03). Na+ channel characteristics were significantly altered by hypoxia. For example, the steady-state inactivation curve was significantly more positive in CYC than in Con (−60 ± 6 mV, n = 8, for CYC and −71 ± 3 mV, n = 14, for Con, P < 0.04). The time constant for deactivation (τd) was much shorter in CYC than in Con (at −100 mV, τd 0.83 ± 0.23 ms in CYC neurons and 2.29 ± 0.38 ms in Con neurons, P = 0.004). We conclude that the increased neuronal excitability in mice treated with cyclic hypoxia is due to alterations in Na+ channel characteristics and/or Na+ channel expression. We hypothesize from these and previous data from our laboratory (Gu XQ and Haddad GG, J Appl Physiol 91: 1245–1250, 2001) that this increased excitability is a reflection of an enhanced central nervous system maturation when exposed to low O2 conditions in early postnatal life. 

Na+ channels; excitability; O2 deprivation

A NUMBER OF CONDITIONS and disease states are associated with tissue hypoxia that is intermittent in nature. For example, one of the important aspects of obstructive sleep apnea/hypoventilation syndrome (OSAHS) in both children and adults is a cyclic hypoxia that results from upper airway obstruction. This intermittent hypoxia occurs in OSAHS throughout the night and can repeat itself numerous times in a single night (4). In sickle cell disease, hypoxia and ischemia are also intermittent, and these may lead to major pathophysiological conditions. In cardiac ischemia, intermittent hypoxia and hypoperfusion may occur before total occlusion takes place.

METHODS

Cyclic Hypoxia

A computer-controlled chamber was developed by us for the induction and maintenance of cyclic hypoxia. This chamber (volume = 80 l) held up to 3 mouse cages simultaneously. The time of the on/off cycle durations was automatically controlled by valves and valve drives and an IBM-compatible computer equipped with an analog-to-digital and digital-to-analog converter and software written in BASIC on an IBM compatible computer in our laboratory. O2 concentration in the chamber was measured by an O2 meter (OM200, Camer Instrument), monitored and corrected at 1 Hz, and recorded twice every minute. Mice were placed in the chamber at the age of 2–3 days with their dam. Cyclic hypoxia was induced daily for 8 h during the day; mice breathed in room air for 16 h at night. Each hypoxic duration in each cycle was ~1.5–2 min with a nadir of fractional inspired O2 of about 7.5%; the “off-time” (normoxia) consisted of ~3 min (Fig. 1).

The effects of cyclic hypoxia on neural properties are not clear. Although there has been a substantial literature on cellular and molecular mechanisms of adaptation to low O2 conditions (see reviews, Refs. 3, 10, 14, 24) and the mechanisms of injury (see reviews, Refs. 2, 6, 8, 9, 13, 20, 22), there are many unanswered questions, especially in relation to the role of hypoxia. For example, it is controversial as to whether a previous exposure to hypoxia renders neurons more or less susceptible to subsequent hypoxic stress (17). Because repetitive hypoxia can be severe and can occur over prolonged periods of time, it may produce central neuronal sublethal damage. It is possible, however, that central neurons may adapt during exposure, suffer less injury, and survive. Because the impact of cyclic hypoxia may depend on neuronal development and the magnitude and extent of exposure to hypoxia, we have studied, in this work, mice in early life and exposed them to a low O2 paradigm for the first 2 wk of life. We used electrophysiological methods to determine the cellular mechanisms that were altered in response to cyclic hypoxia in young mice. We focused our work on the hippocampus because 1) this has been a well-studied region and 2) we have done some of our previous work on hippocampal neurons (7).
Mice were exposed to cyclic hypoxia for about 10–14 days until the time of death. Twelve mice were exposed to hypoxia and thirteen others (from different litters) were used for controls. All exposed mice survived the period of exposure. Pregnant mice (CD1) were all purchased from Charles River.

Preparation of CA1 Cells

Mice at 12–16 days of age were used and killed after inhalation of halothane, and their hippocampi were removed and sliced into 7–10 transverse sections of 400 μm in thickness. The slices were immediately transferred to a container with 2.5 ml of fresh, oxygenated, and slightly stirred HEPES buffer at room temperature. After 30 min of exposure to trypsin (0.08%) and 20 min of protease (0.05%) digestion, the slices were washed several times with HEPES buffer and left in oxygenated solution. The CA1 region was then dissected out and triturated in a small volume (0.25 ml) of HEPES buffer. The CA1 cells studied were obtained from 12- to 14-day-old mice.

Electrophysiological Recording and Solutions

Electrodes for whole cell recording were pulled on a Flaming/Brown micropipette puller (model P-87, Sutter Instrument) from filamented borosilicate capillary glass (1.2-mm OD, 0.69-mm ID; World Precision Instruments or Warner Instrument). The electrodes were fire-polished, and resistances were 2–5 MΩ for voltage-clamp experiments and 7–9 MΩ for current-clamp experiments in the solutions listed below. Membrane potentials (V_m) and action potentials (APs) were recorded in the current-clamp mode. Input resistance (R_in) was measured at −70 mV as 1/slope of the current trace evoked by a ramp voltage from −160 to 100 mV in the voltage-clamp mode. The slope was derived from least-square regression analysis for 100 data points between voltage and current. Current traces in voltage clamp were leak-subtracted. Junction potentials were nullled for each individual cell with the Axopatch 1C amplifier.

For the current-clamp experiments, the external HEPES solution bathing neurons contained (in mM) 130 NaCl, 3 KCl, 1 CaCl_2, 1 MgCl_2, 10 HEPES, and 10 glucose, and pH was adjusted to 7.4 with NaOH. The pipette solution contained (in mM) 138 KCl, 0.2 CaCl_2, 1 MgCl_2, 10 HEPES (Na⁺ salt), and 10 EGTA, and pH was adjusted to 7.4 with Tris. The external solutions for the voltage-clamp experiments contained similar reagents as in the current-clamp experiments, except for adding 10 mM TEA chloride, 5 mM 4-aminopyridine (4-AP), and 0.1 mM CdCl_2 and reducing NaCl from 130 to about 117 mM. The internal pipette solution for the voltage-clamp experiments was also similar to the internal solution for the current-clamp experiments, except for the use of either CsF or CsCl instead of KCl [we had previously shown that there was no difference in whole cell Na⁺ current recorded when CsF or CsCl was used (16)]. The HEPES-buffered solutions for the enzymatic preparation and trituration of the CA1 cells contained (in mM) 125 NaCl, 3 KCl, 1.2 MgSO_4, 1.25 Na_2HPO_4, 30 HEPES, and 10 glucose. Osmolarity of all solutions was adjusted to 290 mosM. All recordings were performed at room temperature (22–24°C). All experiments were performed on 8–27 cells for either the control or cyclic hypoxic group. One-tailed Student’s t-test was performed for comparisons. Values in the text are given as means ± SE. All chemicals were purchased from Sigma.

Recording Criteria

These criteria have been previously used, as detailed in our previous publications.

Morphological criteria. CA1 cells were used if they had a smooth surface, a three-dimensional contour, and were pyramidal in shape. Similar criteria have been used by us (5, 7) and others (11) on freshly triturated neurons. The CA1 cells studied were obtained from 12- to 14-day-old mice.

Electrophysiological criteria. 1) Neurons were considered for recording if the seal resistance was >5 GΩ. 2) Only neurons with a holding current of <0.1 nA (command potential −100 mV) were used in the study. 3) Series resistance was <10 MΩ in neurons studied. The series resistances were compensated at 90% level with the Axopatch 1C amplifier (Axon Instruments). Under these conditions, the error caused by uncompensated series resistances was <1.7 mV. To obtain adequate voltage clamp and minimize the space-clamp problem, only small neurons with short processes were used in Na⁺ current measurements. In addition, we...
only used cells with current-voltage (I-V) curves that were smoothly graded over the voltage range of activation (approximately −50 to −10 mV), as we have done in the past (5, 7, 16).

RESULTS

Body Weight, Brain Weight, and Hippocampal Weight

We measured body, brain, and hippocampus weight (Table 1) to follow the effect of cyclic hypoxia on development. Although body weight was significantly lower in mice exposed to cyclic hypoxia, neither brain weight, hippocampus weight nor the ratio of brain/body weight, hippocampus/body weight, and hippocampus/brain weight were significantly different.

Neuronal properties and membrane excitability. All control (Con) (n = 7) and cyclic hypoxia-exposed (CYC) (n = 9) CA1 neurons fired APs when they were held at −75 mV and given depolarizing currents in the current-clamp mode. No Con and CYC neurons, however, fired spontaneous APs. CYC CA1 neurons had a similar V_m to those of Con neurons (−28 ± 3, n = 8 for CYC vs. −29 ± 3, n = 12 for Con), but they had a significantly lower R_m (568 ± 86 MΩ, n = 16 for CYC vs. 875 ± 150 MΩ, n = 15 for Con neurons, P = 0.04). To determine the excitability of Con and CYC neurons, we systematically determined the rheobase. Stepping all neurons from the same V_m (−75 mV) in the current-clamp mode, the amount of current required to generate one AP was 54 ± 24 pA, n = 8, in CYC but more than double that in Con neurons (117 ± 18 pA, n = 18, P = 0.03; Fig. 2) (Table 2).

Na⁺ Current Magnitude

Because I) neuronal excitability is largely dependent on the characteristics and magnitude of Na⁺ channels, and 2) CYC and Con neurons showed differences in excitability even when neurons were stepped from the same V_m, we examined the Na⁺ channel properties in both groups of neurons. Under voltage clamp, steps from a holding potential of −130 to −20 mV evoked an inward current that reached a peak in <1 ms and decayed quickly to zero current. Tetrodotoxin (TTX; 1 μM) blocked the current almost totally. Based on these characteristics, we considered this current to be a voltage-sensitive fast Na⁺ current. The average peak Na⁺ current in CYC neurons was 1,749 ± 362 pA, n = 18, compared with a much smaller peak Na⁺ current in Con of 634 ± 78 pA, n = 27, (P = 0.0004, Fig. 3A). Because the difference in current magnitude between both groups of neurons could be related to neuronal surface area (as indicated by whole cell capacitance measurements with 3.6 ± 0.3 pF, n = 27, in Con neurons and 5.4 ± 0.5 pF, n = 18, in CYC neurons, P = 0.001), we normalized the peak current to capacitance. Thus the estimated cell sizes would be 219.2 ± 29.3 μm² for Con and 396.1 ± 49.6 μm² for CYC (assuming neurons are all spheres and assuming ~1 μF/cm² specific capacitance). The Na⁺ current density was also much larger in CYC neurons (333 ± 84 pA/pF, n = 18) compared with Con neurons (192 ± 20 pA/pF, n = 27, P = 0.03; Fig. 3B) (Table 2).

Na⁺ current characteristics. We next examined the characteristics of the Na⁺ current to determine whether there are other differences between CYC and Con neurons that could be important in determining excitability.

Activation. With CA1 neurons held at −130 mV, depolarizing voltages were given from −70 to +80 mV with 10-mV increments. For both groups of neurons,

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Table 1. Comparison of cyclic hypoxia treatment on weights

<table>
<thead>
<tr>
<th></th>
<th>Age, day</th>
<th>Body Weight, g</th>
<th>Brain Weight, g</th>
<th>Hippocampal Weight, g</th>
<th>Brain/Body Weight</th>
<th>Hippocampal/Body Weight</th>
<th>Hippocampal/Brain Weight</th>
</tr>
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<tbody>
<tr>
<td>Con</td>
<td>14.7 ± 1.2</td>
<td>9.15 ± 0.44</td>
<td>0.408 ± 0.32</td>
<td>0.016 ± 0.001</td>
<td>0.045</td>
<td>0.0017</td>
<td>0.038</td>
</tr>
<tr>
<td>CYC</td>
<td>14.0 ± 0.5</td>
<td>8.20 ± 0.28a</td>
<td>0.418 ± 0.046</td>
<td>0.015 ± 0.001</td>
<td>0.051</td>
<td>0.0019</td>
<td>0.036</td>
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Values are means ± SE (n = no. of animals or regions). Con, control; CYC, exposed neurons. *Significantly different (P = 0.047) from control.

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Fig. 2. Voltage traces with evolved action potentials (APs) in control (Con, A), CYC (exposed; B) neurons, and mean rheobase (C). In A and B, evoked APs were collected in the current-clamp mode at −75 mV with 10 depolarizing currents, starting with 100 pA and using 10-pA increments in A and starting with 10 pA and using 10-pA increments in B. Voltage traces in A and B start at the bottom and follow upward incrementally. Scales represent 50 mV and 25 ms. In C, the minimum currents used to evoke an AP were averaged. *Significantly different means (P = 0.03).
the threshold for Na\(^+\) channel activation was \(-60\) mV, and the Na\(^+\) current reached a peak at \(-20\) mV. The midpoint for the voltage-conductance relation \((g/g_{\text{max}})\) was about the same in both groups (\(-35 \pm 3\) mV, \(n = 10\), for Con neurons, and \(-41 \pm 3\) mV, \(n = 9\), for CYC neurons). Similarly, the activation slope factors, \(3.1 \pm 0.6\) for Con and \(3.3 \pm 0.5\) for CYC, were not different in both groups of neurons (Fig. 4). Although there was no difference between the two groups either in the midpoint of voltage-conductance curves or slope factors, at some particular voltages, \(-30\) mV, for example, \(g/g_{\text{max}}\) was statistically different \((P = 0.02)\) between the Con \((0.56 \pm 0.10, n = 10)\) and CYC \((0.86 \pm 0.06, n = 9)\) groups.

Steady-state inactivation. Steady-state inactivation of the Na\(^+\) current was studied using a prepulse protocol with increased interval durations between the two pulses. Although the time constant for recovery (\(\tau_h\)) was not much different for both groups (\(2.3 \pm 0.6\) ms, \(n = 9\), and \(2.8 \pm 0.7\) ms, \(n = 11\), for CYC and Con neurons, \(P = 0.29\)), there were significant differences if two pulses were below about 5 ms apart. For example, when the time \((t)\) between the two pulses was \(2.6\) ms, the ratio of the peak of the second current to that of the first \((I_{\text{peak2}}/I_{\text{peak1}})\) for Con was \(0.16 \pm 0.008\) and \(0.53 \pm 0.09\) for CYC \((P = 0.002)\). At \(t = 5.12\) ms, the ratio \(I_{\text{peak2}}/I_{\text{peak1}}\) for Con was \(0.52 \pm 0.08\) and \(0.74 \pm 0.07\) for CYC \((P = 0.02)\) (Fig. 6C).

Deactivation characteristics. We also examined the deactivation properties, i.e., the transition from the open to the resting closed state for both groups of neurons. We held CA1 neurons at \(-100\) mV, depolarized them for \(1\) ms to \(-10\) mV, and repolarized to \(-100\) mV (Fig. 7, A and B). Current traces in response to this protocol are shown separately in Fig. 7, A (Con) and B (CYC), and superimposed in Fig. 7C. The normalized current traces for both Con and CYC are superimposed in Fig. 7D. Notice that Fig. 7C represents the actual recordings overlaid, whereas Fig. 7D represents scaled recordings. The averaged time constant for deactivation, \(\tau_d\) at \(-100\) mV, was significantly smaller for CYC neurons \((0.8 \pm 0.2\) ms, \(n = 9)\) than that for Con neurons \((2.3 \pm 0.4\) ms, \(n = 14, P = 0.004)\) (Fig. 7E). At \(-70\) mV, the results showed a similar difference \((1.9 \pm 0.6\) ms, \(n = 9,\) for CYC and \(3.1 \pm 0.5\) ms, \(n = 14,\) for Con) (Table 2).

![Fig. 3. Na\(^+\) current \((I_{\text{Na}\text{-p}})\) amplitude (B), Na\(^+\) current density (C) and examples of current traces from both Con and CYC neurons (A, overlaid). Peak Na\(^+\) currents were obtained in voltage clamp, and current densities were derived from the ratio of peak Na\(^+\) current over whole cell capacitance. *Statistical significance between means \((P = 0.0004\) for B and \(P = 0.03\) for C).](http://ajpcell.physiology.org/)
DISCUSSION

In this study, we have found that CA1 neurons are more excitable in mice exposed to cyclic hypoxia. Our results also suggest mechanisms that can explain the differences in excitability between CYC and Con CA1 neurons.

In spite of the fact that CYC neurons have a lower input resistance, they still exhibited a lower rheobase for excitability. There are several reasons for the increase in excitability of CYC neurons. First, the Na⁺ current density was significantly (73%) higher in the exposed neurons than in control counterparts. Second, the steady-state inactivation curve was significantly shifted in the depolarizing direction, i.e., there were more channels available for recruitment in CYC than in Con neurons, especially at physiological voltages, such as between −40 and −80 mV. Third, the probability of available channels after depolarizations was higher in CYC than Con neurons, especially in the immediate period after stimulation. Fourth, it is also possible that the faster deactivation time constant of the Na⁺ channels in CYC neurons is a factor that allows these neurons to be more readily recruitable than control neurons.

Because our data were collected in the whole cell configuration and these results demonstrated that the whole cell Na⁺ current was larger in CYC than that in Con neurons, we cannot presently differentiate between the various reasons that led to the increased whole cell current. For example, the Na⁺ channel expression in the plasma membrane may be higher upon exposure to cyclic hypoxia. Alternative possibilities are that the single channel ionic conductance and the open probability of the channel may have been increased.

One additional interesting finding in this work was that the capacitance in CYC neurons was significantly larger than that in Con neurons. Although there could have been a bias in the selection of these cells, it is possible that cells in CYC mice were larger than their counterparts in Con mice. This may not be surprising because other investigators have shown that repeated hypoxia induces structural changes in hippocampal...

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Fig. 4. Activation and voltage-conductance relationship of the Na⁺ current. In A and B, current traces were collected from −70 to 0 mV with 10-mV increment from a holding potential of −130 mV for Con and CYC. In C, the current-voltage (I-V) relationship was expressed as peak Na⁺ current at each resting membrane potential (I/I ratios) against the Vm. In D, the voltage-conductance relationship was expressed as g/gmax against the Vm. Curves were fitted by the Boltzmann equation. *Significantly different means (P < 0.02) (see text).

Fig. 5. Steady-state inactivation of the Na⁺ current. In A and B, current traces were collected at −20 mV from prepulse potentials ranging from −130 to −20 mV with an increment of 10 mV and a duration of 502 ms. Voltage protocol is shown under the trace of A. The scales in A and B represent 1 nA and 10 ms. I/I ratios were plotted against the prepulse potential in C. Curves were fitted by the Boltzmann equation.
CA1 neurons, such as a reduction in the number of dendritic branching (21). Hence, it would be useful at a later stage to characterize the morphological changes in these neurons quantitatively because these alterations, such as reduction in the number of dendritic branching and increase in cell size, may have profound effects on cell-cell communications.

We have previously published data on the effect of cyclic hypoxia in mice after about 4 wk of exposure. Our previous data showed that the animals exposed to cyclic hypoxia had a much lower excitability than those that were not exposed; their Na\(^{+}\)/H\(^{+}\) currents were also downregulated compared with control animals (7). Our data in this work would strongly suggest that the maturation of the in vivo exposed neurons over the first month of life in mice is quite different from that of the control, nonexposed neurons. Indeed, there are two main ideas that we highlight in this work. First, the paradigm for the hypoxia exposure in vivo is very important. Here, we observed that 2 wk of the cyclic hypoxic stress induced a very different profile from the same stress for 4 wk. Second, the maturation in early life of certain membrane proteins, such as the Na\(^{+}\) channels, in terms of properties and regulation/expression, depends on environmental oxygenation and on the length of hypoxic exposure.

![Fig. 6. Recovery from inactivation of the Na\(^{+}\) current. In A and B, 2 identical pulses were delivered with increasing intervals (t) in between each pair of pulses. Voltage protocol is shown under the traces of A. The scales in A and B represent 1 nA and 10 ms. In C, recovery from inactivation of the Na\(^{+}\) current was plotted as the ratio of the peak of the second current over that of the first (I_{peak2}/I_{peak1}) against the intervals in a 2-pulse voltage protocol.](image)

![Fig. 7. Deactivation of the Na\(^{+}\) current. In A and B are traces representing deactivation of the Na\(^{+}\) current in Con and CYC neurons. The voltage protocol is shown under the traces of A. C: current traces from A and B are superimposed. D: normalized current traces for both Con and CYC are superimposed. E: the averaged time constants for deactivation (τ_d) as repolarized to −100 mV are plotted for the Con and CYC groups. The scale in B is applicable for A, B, and C and represents 1 nA and 10 ms. *Significantly different means (P = 0.004) for τ_d as repolarized to −100 mV.](image)
The changes in Con neurons reflect developmental changes of Na\(^+\) channels characteristics during normal maturation, as we and others have demonstrated in rodents (1, 12, 18, 23). In summary, neurons obtained from exposed mice seem to “mature” in vivo at a faster rate when exposed to cyclic hypoxia early in life in the first 2 wk. However, this maturation seems to halt and, by about 28 days, the phenotype of the exposed mice had lagged behind (Fig. 7).

One question that can be raised from our current work is whether the changes observed in mice after the first 2 wk of exposure reflect an adaptive strategy that could benefit the overall survival of these neurons and possibly the animal itself. Although it is difficult to be certain, it would be intriguing to speculate that the increase in Na\(^+\) channel and increase in excitability in the first few weeks of life in hypoxia is adaptive and beneficial to the organism. It has been shown that the enhancement of Na\(^+\) channels in early life (5) is critical in the process of synaptogenesis and the refinement of synaptic connections (19). Hence, it is possible that hypoxia in early life enhances synaptic connectivity. This, in turn, will enhance brain development and can counteract the potential hypoxia-induced metabolic depression and its negative impact on brain development.

Although hypoxia could have directly affected neuronal excitability (16), it could also have induced the neuronal changes indirectly via other means. For example, maternal stress could have affected growth and development of the litter pups. We do not believe, however, that this is the case from previous experiments done by Mortola et al. (15). In addition, similar experiments that we had done in our laboratory on rat pups showed that foster mothers raised in normoxia (the majority of the day) showed that the effect on body size was related to hypoxia rather than maternal effects (unpublished observations). Other factors that could have played a role include alterations in the hormonal milieu, extracellular pH, neurotransmitter release, or growth factors in the microenvironment of neurons.

In summary, we have shown that the younger mice exposed to cyclic hypoxia have an overall higher hippocampal neuronal excitability. This increased excitability can be explained by 1) an upregulation of the Na\(^+\) current and 2) alterations in the channel characteristics, including activation, steady-state inactivation, recovery from inactivation, and deactivation. This investigation documents the existence of an important functional link between cyclic hypoxia and the voltage-sensitive Na\(^+\) channel. The importance of this link is related to the idea that the changes in the Na\(^+\) channel documented in this work may be critical for the increased excitability of neurons and their metabolic demands in their early life.

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