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O_2 DEPRIVATION INDUCES A MAJOR DEPOLARIZATION IN BRAIN STEM NEURONS IN THE ADULT BUT NOT IN THE NEONATAL RAT

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SUMMARY

1. In order to study the neuronal response to hypoxia with maturation, hypoglossal neurons from adult and neonatal rat (3–7, 14–16, 21 and 28 days) brain stem slices were subjected to O_2 deprivation. All neurons depolarized and showed no evidence of hyperpolarization at any time during the hypoxic period.

2. The magnitude of depolarization was about three-fold larger in adult hypoglossal neurons (mean = 32·0 mV) than in young neonatal neurons (mean = 10·4–11·2 mV) during hypoxic exposure (15–20 Torr) of 5 min. During longer periods of hypoxia of 15–30 min, neonatal cells showed an increase in the magnitude of depolarization reaching a level close to 80% of that in the adult.

3. In the early phase of hypoxia, adult neurons increased peak and steady-state spike frequency to induced current injections. Later, both spike frequencies decreased and, in 1/2 of adult neurons, there was a depolarization block. Input resistance (R_N) of most adult neurons increased during hypoxia (R_N = 180% of control after 5 min). Though neonatal neurons increased firing frequency, none had depolarization block and there was no increase in R_N.

4. Tetrodotoxin (TTX), tetraethylammonium (TEA), apamin, high Mg^{2+}/low Ca^{2+} solutions and intracellular ethyleneglycol-bis-(β-aminoethylether)*N,N,N',N'*-tetracetic acid (EGTA) did not reduce the magnitude of depolarization in hypoglossal neurons of 4-week-old and adult rats. Strophanthidin application depolarized hypoglossal neurons but decreased R_N.

5. Ion-selective electrodes used to measure K^+ concentrations in the extracellular fluid (K_o^+) revealed a major increase in K_o^+ (mean = 3·2 mM) in the adult hypoglossal area but not in the newborn tissue (mean = 0·65 mM). This probably reflects a difference in the amount of K^+ efflux between neonatal and adult hypoglossal neurons. Shrinkage in the extracellular compartment in the adult may also account for some of the difference.

6. These results suggest that neonatal neuronal tissue is more tolerant to hypoxia than the adult, with the inherent cellular properties being maintained in newborn but not in adult neurons. The difference in membrane depolarization during hypoxia between the neonate and the adult can be attributed, in part, to differences in K_o^+. The reason(s) for the difference in K_o^+ is not known but could be due, to some extent, to different rates of intracellular ATP depletion and failure of the Na^+–K^+ pump.

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INTRODUCTION

A common feature of all mammalian neurons is their sensitivity to hypoxia. Hypoxia sensitivity of adult cortical cells is manifested by a rapid loss of electrical activity (Fujiwara, Higashi, Shimoji & Yoshimura, 1987; Leblond & Krnjević, 1989). This reduced activity may be an inherent property of cortical cells since *in vitro* intracellular recordings have demonstrated that adult hippocampal neurons hyperpolarize in response to hypoxia (Fujiwara *et al.* 1987; Krnjević & Leblond, 1989; Leblond & Krnjević, 1989).

In contrast to cortical neurons, much less is known of the response of neurons in other brain regions, such as the brain stem. Since a large number of brain stem neurons subserve autonomic functions, it would be important to determine whether these cells, like cortical neurons, do hyperpolarize and shut off their activity in response to hypoxia. Indeed, brain stem neurons may jeopardize further cardiorespiratory function if they were to be silenced by O_2 deprivation. For instance, if sympathetic neurons rapidly hyperpolarized in response to hypoxia, hypotension and hypoperfusion may exacerbate the effects of primary hypoxia. Similarly, brain stem respiratory neurons, such as hypoglossal neurons, may not be able to recruit the genioglossus muscle and relieve obstructive apnoea, if hypoxia, secondary to apnoea, were to have a hyperpolarizing effect. Despite the importance of brain stem neurons in maintaining cardiorespiratory homeostasis, no direct information is known of the effects of hypoxia on these cells.

It has been shown that the newborn has a different respiratory responsiveness to hypoxia than the adult (Haddad & Mellins, 1984). Also, it has long been observed that newborn mammals survive a nitrogen environment for longer periods of time than adult animals and that survival time depends on postnatal age and species maturity at birth (Kabat, 1940; Fazekas, Alexander & Himwich, 1941). The mechanism for this hypoxia tolerance is not known, but it has been recently suggested that the difference in tolerance can be attributed to differences in membrane properties between neonatal and adult central nervous system neurons (Hochachka, 1986).

In the present experiments, we address three questions: (1) what are the electrophysiological responses of adult brain stem respiratory neurons to hypoxic conditions, (2) do these responses change with development in early life, and (3) what are some of the mechanisms underlying the hypoxia-induced changes in neuronal function? In order to answer these questions, we used a brain stem slice preparation with intracellular recordings. Adult and newborn rats were chosen since rats are relatively immature at birth (Fish & Winick, 1969) and maturational patterns, if present, are more likely to be detected in the rat than other species. We focus in this work on hypoglossal neurons since these innervate important upper airway muscles and since they are relatively large neurons (Haddad, Donnelly & Getting, 1990). Some of the current data and results have been reported briefly in abstract form (Haddad & Donnelly, 1989; Donnelly & Haddad, 1989).

METHODS

Hypoglossal neuron identification

Hypoglossal identification was undertaken by placing horseradish peroxidase–wheatgerm agglutinin crystals on cut hypoglossal nerves of anaesthetized (methoxyflurane) adult ($n = 8$) and 7-day-old rats ($n = 3$). Two days later, the animals were deeply anaesthetized with methoxyflourane and intraperitoneal injection of pentobarbitone (65 mg). The thorax was then opened and cold intracardiac saline was infused followed by 4% formaldehyde in phosphate buffer solution. The brain stems were removed and cut into 50 μm sections. These sections were reacted for the presence of HRP using tetramethylbenzidine, counterstained with Neutral Red and observed under light and dark field illumination. The location of HRP-stained neurons was subsequently used for determining the microelectrode recording sites.

Preparation of brain stem slices

The methods used for brain stem slice preparation have been presented previously in detail (Haddad & Getting, 1989) and will be described only briefly here. Adult rats (weight = 175–225 g) were lightly anaesthetized by inhalation of methoxyflurane and decapitated. Three 400 μm thick transverse slices were obtained between 300 μm rostral to obex and 600 μm caudal to obex. These slices were placed in a chamber and allowed to equilibrate for about 20–30 min. The time between decapitation and placement of the slices in the recording chamber was within 6·5 min. The temperature in the chamber was controlled and maintained at 35–36 °C. Flow of the oxygenated Ringer saline (95% O_2–5% CO_2) was 2–3 ml/min and slices were kept subfused throughout the experiment. The same gas that oxygenated the saline also flowed over the slices after being warmed and humidified. Ringer solution pH was 7·4 after equilibration with 5% CO_2 and contained in mM: NaCl, 125; $MgSO_4$, 1·3; $CaCl_2$, 2·5; $NaHPO_4$, 2·5; $NaHCO_3$, 26; glucose, 10. KCl was varied from 3·0 to 6·2 mM to enhance spontaneous spiking activity. Brain stem slices remained viable for at least 6–8 h. Brain stems from neonatal rats were prepared in the same way. The number of cells studied at each age is: $n = 32$ at 3–7 days, $n = 15$ at 14–16 days, $n = 14$ at 21 days, and $n = 8$ at 28 days. Experiments were also done on twenty-two adult brain stem neurons.

Electrophysiological studies and protocol

Membrane potential was recorded using glass microelectrodes filled with 3 M-KCl (resistance = 50–100 MΩ). Data were accepted for analysis only if (1) the height of the action potential was over 70 mV and (2) the resting membrane potential was stable and at least −50 mV.

Our protocol included three phases. The first determined the active and passive properties of the neuron being studied during baseline, i.e. before hypoxia. The second phase determined the neuronal properties during 5 min of hypoxic exposure and the third was recovery. Hypoxia was induced by switching the perfusate to one equilibrated with 95% N_2–5% CO_2 and by stopping all gaseous O_2–CO_2 over the slices. Drugs were applied by switching perfusion solutions. Tetrodotoxin (TTX) (0·3–1 μM), tetraethylammonium (TEA) (10–20 mM), apamin (50 and 100 pM), strophanthidin (20 and 50 μM; Kocsis, Malenka & Waxman, 1983), low Ca^{2+}/high Mg^{2+} solutions (0·5 mM-Ca^{2+} and 3·5 mM-Mg^{2+}) and zero Ca^{2+} solutions with cobalt chloride ($CoCl_2$, 1 mM) were used. In solutions containing zero calcium with $CoCl_2$, no phosphate or bicarbonate was added to avoid precipitation; Trizma (15 mM) was used as a buffer. All these solutions bathed the slices for at least 20–30 min before hypoxic exposure was attempted and evaluated. Intracellular EGTA was applied by ionophoresis through the recording electrode (−0·5 to −1·0 nA for 15 min) before hypoxia.

Before hypoxia was instituted, a series of depolarizing and hyperpolarizing pulses of increasing intensity was performed to examine the input resistance and study the repetitive firing properties of neurons under consideration. Depolarizing pulses lasted 2 s and hyperpolarizations 200 ms each. During the hypoxic period and recovery, each cell was subjected to pairs of hyperpolarizing (−1·0 to −2·0 nA) and depolarizing (approximately 110% of control rheobase) pulses every 15 s in order to examine the changes in cellular properties during hypoxia as a function of time. In order to study the repetitive firing properties during hypoxia, the interspike interval period was converted to instantaneous frequency and plotted against time for each current pulse. Spike frequency adaptation (SFA) was quantified by dividing steady-state frequency (usually the final 1 s stimulus period) by peak frequency × 100 (SFA index).

Ion-selective electrodes

Electrodes were fabricated from 1·0 mm borosilicate glass with a solid filament. After pulling such glass electrodes (tips 1–2 μm), the back end of each electrode was exposed to dimethyldichlorosilane vapour for about 40 min and baked for 1 hr at > 100 °C. A small volume of K^+ liquid ion-exchange resin (LIX; Corning 477317) was injected to fill the top. The remainder of the electrode shaft was backfilled with chloride containing solution (0·1 M-KCl) and mounted in a holder filled with KCl. Electrode potential was measured using an electrometer (WPI, FD 223) and a reference electrode filled with 3 M-KCl. Calibration of the electrode was done by measuring the change in voltage at 1, 5, 10 and 100 mM-KCl. Selectivity of K^+ over Na^+ was more than 50:1. K^+ concentration in the extracellular fluid (K_o^+) was calculated comparing voltages recorded in slices to potentials recorded in calibration solutions.

Three additional experiments were undertaken with another LIX (Fluka ionophore 1, cocktail B) to confirm the observations obtained with the Corning LIX. Using our microelectrodes, this LIX has greater measured selectivity for K^+/Na^+ (1000:1) and is insensitive to acetylcholine or choline (> 1000:1, K^+/Ch) or tetraethylammonium (20:1, K^+/TEA). Using this LIX, we also calibrated these electrodes with two sets of solutions. The first set contains NaCl (149, 145, 140, 50 mM) with varying concentrations of KCl (1, 5, 10, 100 mM) to make a total of 150 mM; in the second set, solutions containing KCl alone were used. These calibration points with K^+ alone included 1, 2, 5, 8, 10, 20 and 100 mM-KCl. Solutions of KCl with NaCl gave readings that were ± 3 mV different from those with KCl alone. This is a small difference compared to that which occurs in the adult slice during hypoxia (e.g. 30–40 mV).

O_2 tension measurements

Multisensor oxygen probes (Ottosensors) utilizing thin-film and solid-state technologies were used to measure tissue O_2 tension. These multisensor probes have a response time of 3–5 s and a sensitivity of 0·5 mmHg of oxygen. Calibration was performed by immersion of the sensor in Ringer solutions equilibrated for 1 h with 100% O_2, 10% O_2 balance N_2 and 100% N_2. To measure P_{O_2} as a function of tissue depth, we used the Diamond General's miniaturized single sensor oxygen probes. A gold cathode is built into a glass microelectrode (3 μm tip diameter) and is covered by a oxygen-permeable membrane at the tip of the electrode. This sensor's time constant is less than 4 s.

Statistical analysis

Analysis of the change in membrane potential and input resistance as a function of time during hypoxia and age was tested using ANOVA and *t* test for *post hoc* testing. Similarly, changes in frequency–time and frequency–current profiles were used to assess changes in excitability and repetitive firing properties. Differences in means were considered statistically significant if $P \leqslant 0.05$.

RESULTS

Location of hypoglossal motoneurons

Hypoglossal motoneurons were found in high concentration between 0·5 mm rostral and 1 mm caudal to obex (Fig. 1). At levels caudal to obex, hypoglossal neurons were ventral and lateral to the central canal. Rostral to obex, hypoglossal neurons abutted on the fourth ventricle and lay just lateral to the midline. Purity of hypoglossal motoneurons in this area was high (> 90%) since very few cells stained with Neutral Red and had no HRP reaction product (Fig. 1).

Tissue P_{O_2}

P_{O_2} was measured as a function of depth in the hypoglossal area of both neonatal ($n = 3$) and adult brain stem slices ($n = 3$). During subfusion with Ringer solution equilibrated with 95% oxygen, tissue P_{O_2} at about 100–200 μm measured about 75 Torr in both adult and neonatal slices. P_{O_2} was always greater than 150–200 Torr in

the more superficial levels (upper 25 μm). The time course of the change in P_{O_2} during hypoxia was quantified at a depth of approximately 100 μm in three additional experiments (Ottosensors). P_{O_2} started to decrease within 8–10 s and dropped to about 50% of its original level; by 30–40 s P_{O_2} reached its nadir (10–15 Torr). The

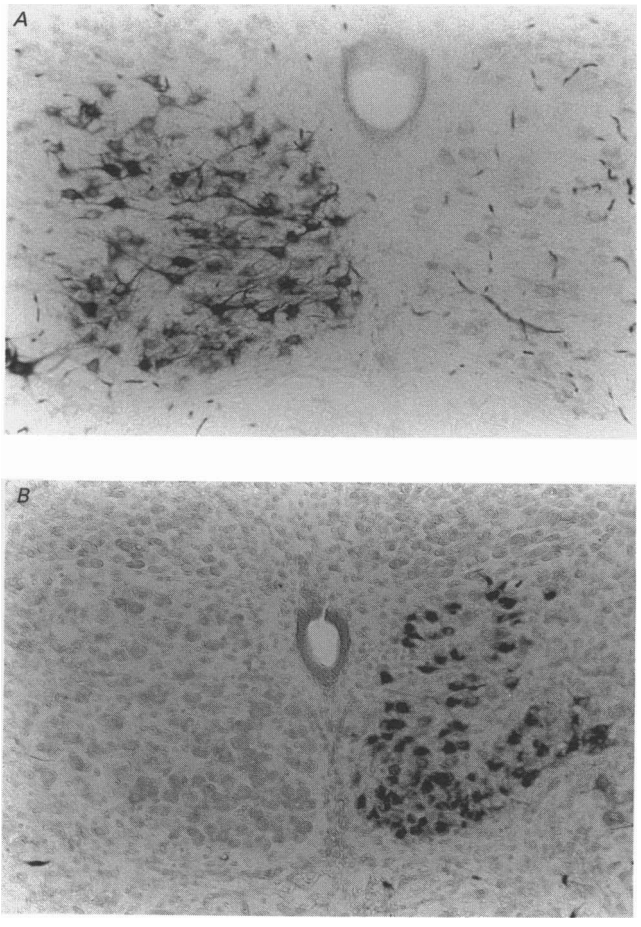

Fig. 1. *A*, light field micrograph showing the location of HRP reaction products after anterograde transport of HRP–WGA from the hypoglossal nerve in an adult rat. Sections are counterstained with Neutral Red. Few cells stained with Neutral Red had not HRP reaction products, indicating that a high purity of hypoglossal motoneurons exists in this area. *B*, section in the newborn (10-day-old). Note that a similar conclusion about purity of motoneurons can be drawn.

return to baseline upon reoxygenation was slower and baseline P_{O_2} was reached after 1–2 min. Similar time course changes were found in both neonatal and adult tissue.

Changes in excitability and membrane potential in adult and neonatal neurons

In contrast to hippocampal CA1 neurons which primarily hyperpolarize in response to hypoxia (Fujiwara *et al.* 1987; Leblond & Krnjevic, 1989), the principal response of hypoglossal neurons to hypoxia was depolarization. This started slowly

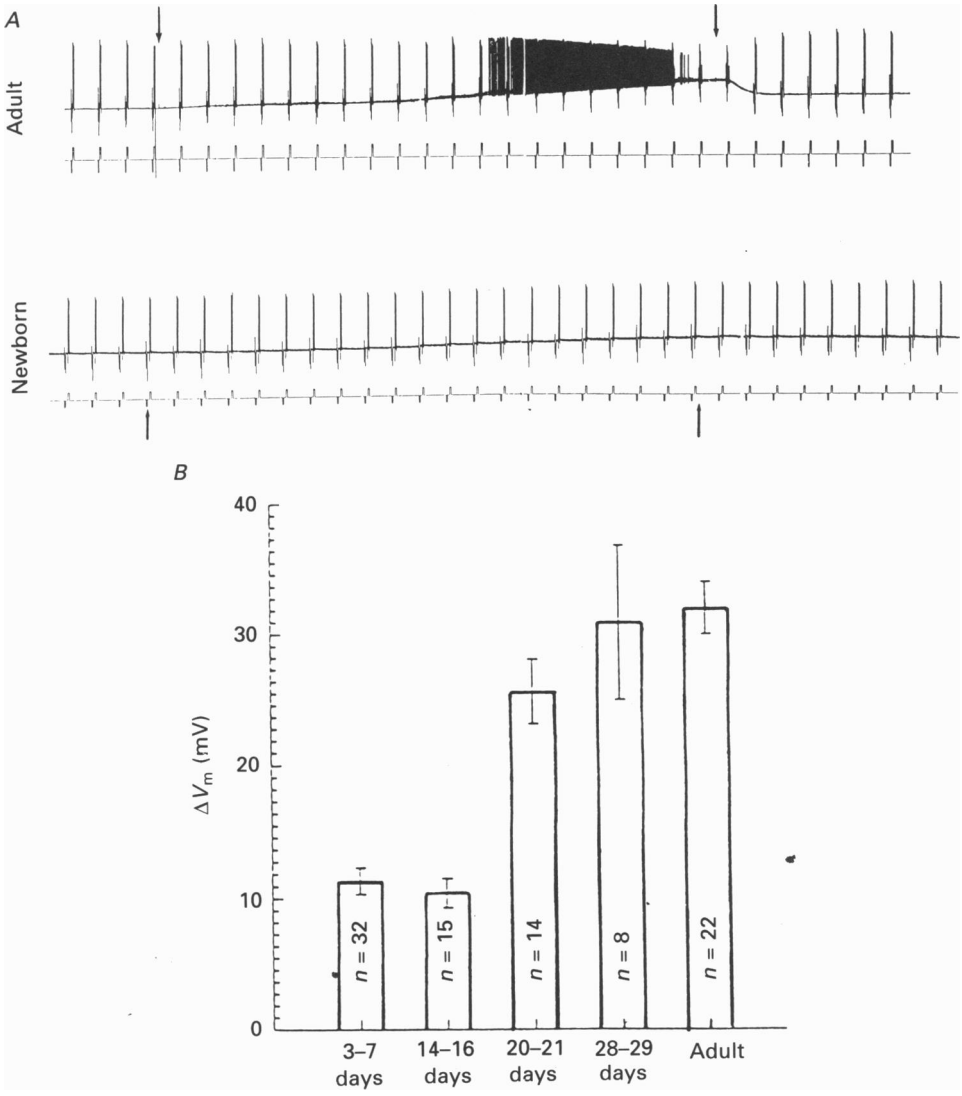

Fig. 2. *A*, adult and neonatal (5-day-old) hypoglossal neuron polygraphic tracings of intracellular potential and injected current applied through the recording electrode during exposure to low tissue oxygen. Note major depolarization in the adult but not in newborn. Membrane potential during control recordings in the adult was −78 mV, −69 mV at 2·5 min, −54 mV at 4 min, and −47 mV at 5 min. Current pulses given were ± 1·8 nA throughout every 15 s. Action potentials were 75 mV in amplitude during control recordings. Arrows mark beginning and end of hypoxia. *B* shows mean ± S.E.M. of maximum depolarization as a function age.

with a latency of 30 s and increased in rate between 1·5 and 3 min (Fig. 2*A*). Most of the depolarization (80–90 %) took place by 4 min. By 5 min, adult hypoglossal neurons had an average maximum depolarization of 32·0 ± 9·6 mV (Fig. 2*B*) with a range of 20–57 mV (Fig. 3, cross-hatched bars). Typically, as neurons depolarized gradually, they became spontaneously active (Fig. 2*A*) and in about half of the adult

neurons, a depolarization block was observed towards the end of the 5 min experimental run (Fig. 2). This block was apparent as neurons ceased to fire and were unresponsive to injected currents. The amount of depolarization with hypoxia did not seem to depend on the original K^+ concentration of the perfusate. Five adult

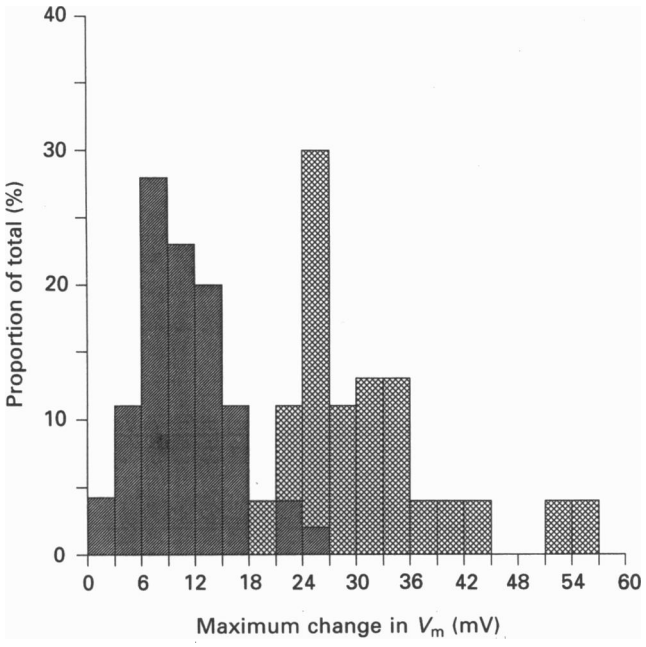

Fig. 3. Histogram distributions of the maximum change in V_m (as a percentage of total) from control to that at 5 min after instituting hypoxia in both, neonatal (filled) (3–7 and 14–16 days) and adult hypoglossal neurons (cross-hatched). Note that the region of overlap (18–27 mV) is small and that the distributions are fairly separate.

hypoglossal neurons studied with an extracellular K^+ of about 3 mM had a maximal change in membrane potential (V_m) with hypoxia that fell within the range obtained with 5–6 mM-K^+.

All adult hypoglossal neurons had an increase in presumed synaptic noise that started 1–2 min into hypoxia. This manifested as an increase in amplitude of post-synaptic potentials (Fig. 4). Towards the end of the experimental run (> 4·5 min), there was a decline in the synaptic noise in the neurons which depolarized most and whose V_m reached low levels (−35 to −30 mV).

Input membrane resistance (R_N), as measured by periodic injections of negative current through the recording electrode, changed during the hypoxic period. Out of ten cells we studied in detail, seven increased their R_N, two others had little change and one decreased its R_N. On average, R_N increased (Fig. 5*A a*) and this increase reached 180% of control by 5 min (Fig. 5*B*). Since hypoxia induced a depolarization and since R_N might have changed as a function of V_m, we argued that the increase in R_N may be secondary to the observed depolarization. In order to address this question, we performed experiments ($n = 5$) in which we measured R_N throughout the 5 min hypoxia period, before and after negative current injection used to increase

V_m to the control level in the discontinuous current clamp mode. We found that hypoxia induced an increase in R_N in four out of five neurons (control = 8·3 ± 2·5 MΩ *versus* hypoxia = 12·0 ± 4·4 MΩ) and this increase in R_N was possibly partially dependent on the change in V_m (R_N during hypoxia without V_m change = 10·7 ± 4·2 MΩ).

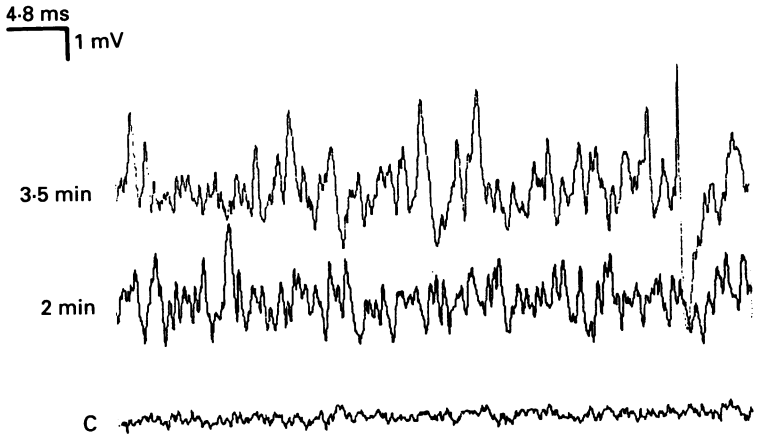

Fig. 4. Plots of synaptic noise of control record (C) ($V_m = -77$ mV), 2 min ($V_m = -73$ mV) and 3·5 min ($V_m = -57$ mV) into hypoxia in an adult hypoglossal neuron. Note the large increase of presumed synaptic noise with hypoxia.

Hypoxia induced an increase in evoked spike frequency rates, particularly in peak compared to steady-state rates (Fig. 6). Note the large increase and the subsequent decline in spike frequency and total elimination of action potentials, late in the hypoxic period. Note also that the peak frequency increased much more than the steady-state frequency. Upon reoxygenation, all adult neurons started to repolarize within 10–15 s (Fig. 2*A*). Action potentials could be induced in all neurons within 1–2 min after reoxygenation, and all electrophysiological changes went back to control levels within 3–10 min.

Hypoglossal neurons in the first 2 weeks of life behaved very differently from adult hypoglossal cells. Young neurons depolarized significantly less than adult neurons (Fig. 2*A*). The mean maximal change in V_m was 11·2 ± 5·4 mV (mean ± S.D.) at 3–7 days and 10·4 ± 4·1 mV at 14–16 days or about one-third that of adult neurons (Fig. 2*B*). The range of the maximal change in V_m for neonatal neurons is shown in Fig. 3. Note that the distribution of the maximal change for neonatal neurons is shifted to the left and that there is only a small overlap between the neonatal and adult populations of neurons. Only three of forty-seven neonatal neurons (up to 16 days) had maximum depolarization values for adult neurons. Neonatal hypoglossal neurons perfused with a K^+ concentration of 3 mM (rather than 5–6 mM) had a maximum change in V_m during hypoxia of 5–10 mV falling within the range obtained with higher concentrations of K^+.

Previous reports have suggested that the change in V_m during hypoxia may be a function of the starting V_m (Leblond & Krnjevic, 1989). Thus, the differences we observed between newborns and adults may have been determined by differences in

starting V_m. In fact, neonatal hypoglossal neurons have a baseline V_m of -69 ± 9 mV (mean $\pm$ S.D.) and adult hypoglossal neurons have a V_m of -80 ± 9 mV. To address this question, a subpopulation of the neurons were analysed based on similar control V_m. We had ten newborn and four adult neurons with a resting V_m of -65 to

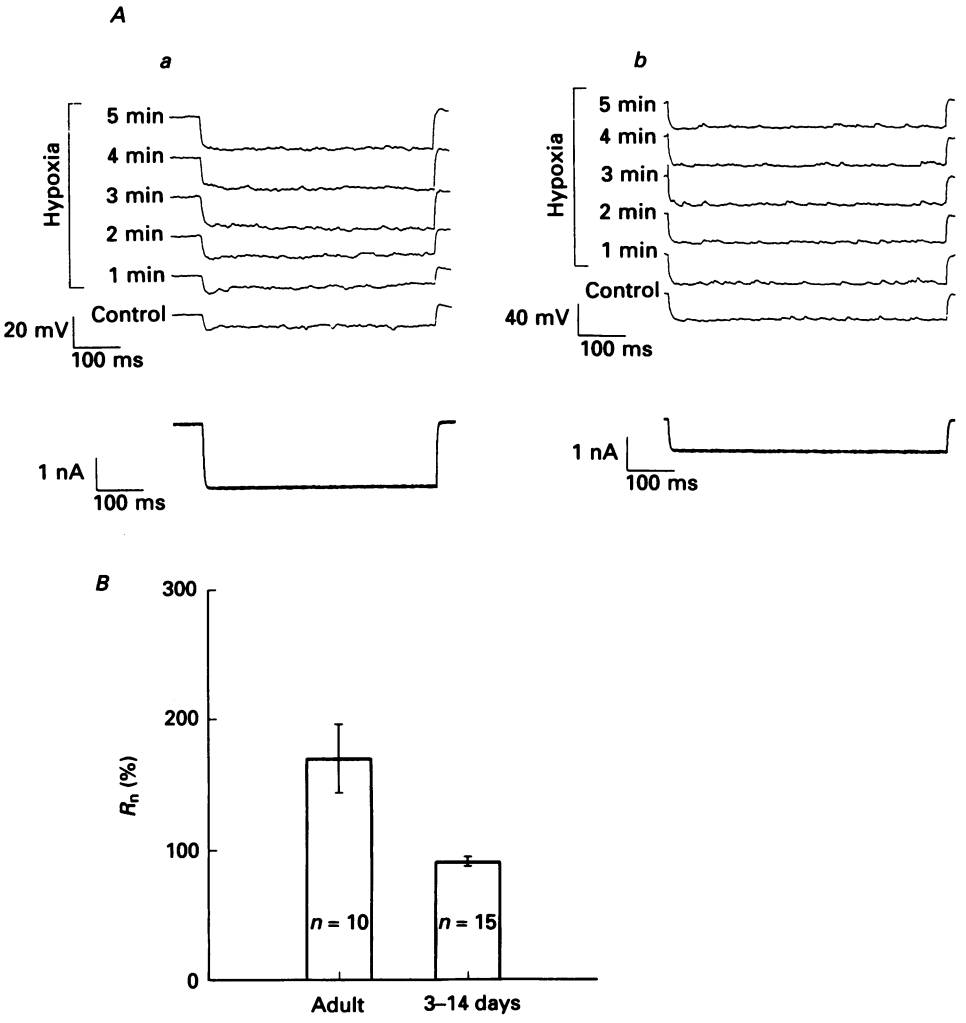

Fig. 5. Changes in R_N during O_2 deprivation. *A a*, voltage tracings before and during -2 nA current pulses applied to an adult hypoglossal neuron. Note increased R_N during hypoxia. *A b*, voltage tracings for newborn hypoglossal neuron. Note lack of change in R_N. *B*, average change $\pm$ S.E.M. in R_N (as percentage of control) after 5 min of low oxygen for adult and newborn hypoglossal neurons. Note R_N was significantly increased for adult but unchanged for newborn. Control R_N: adult, $20{\cdot}8 \pm 3{\cdot}0$ MΩ; 3- to 14-day-old, $9{\cdot}9 \pm 1{\cdot}2$ MΩ.

-70 mV, and these neurons showed a maximum V_m change with hypoxia of 13 ± 5 mV and 38 ± 14 mV, respectively. We also had eight newborn and four adult neurons with control V_m between -70 to -75 mV. Maximum change in V_m during hypoxia was 11 ± 5 and 31 ± 7 mV for newborns and adults, respectively. Hence,

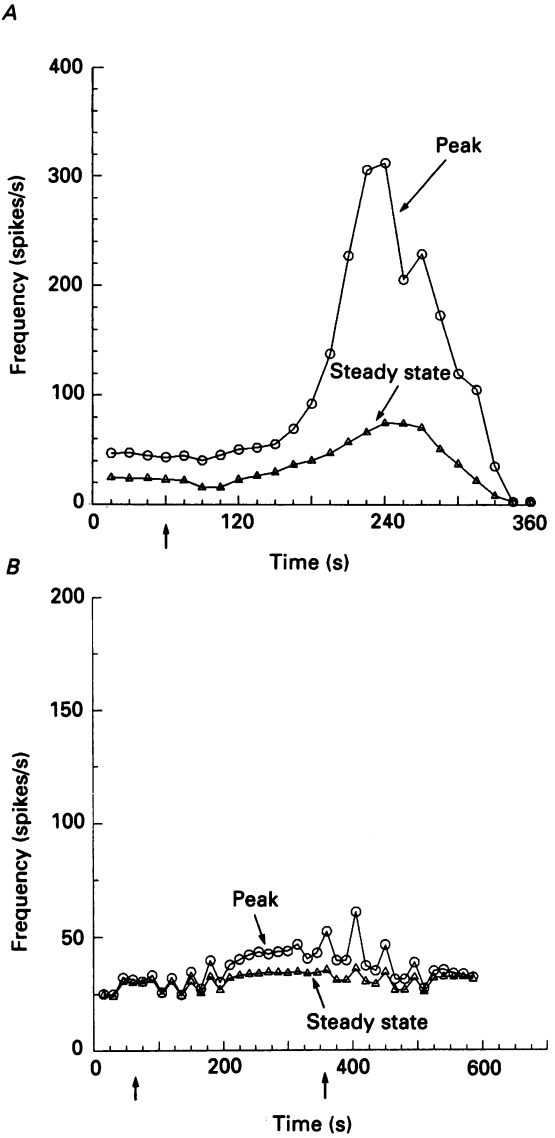

Fig. 6. *A*, action potential frequency–time plot in an adult hypoglossal neuron exposed to hypoxia. Time at arrow represents time of hypoxia induction. Hypoxia was instituted for 5 min. Note the large increase in spike frequency of firing (especially peak) with evoked stimulation during the course of hypoxia. *B*, similar plot for a newborn (6-day-old) hypoglossal neuron. First arrow indicates start of hypoxia and second represents end of hypoxia. Note the small increase in frequency of firing with hypoxia.

despite the similarity in resting V_m, differences in V_m changes persisted during hypoxia between neonate and adult.

Although neonatal neurons also exhibited an increase in synaptic potential, none of them became spontaneously active (Fig. 2). Similarly, R_N was unchanged in newborn cells during the hypoxic period (Fig. 5). With positive current injections,

there was an increase, albeit small, in both peak and steady-state frequency (Fig. 6), and none showed a decline in spike frequency or depolarization block.

Depolarizations of magnitudes close to those in adult hypoglossal neurons could be induced in neonatal neurons by exposure to hypoxia for longer periods of time. In

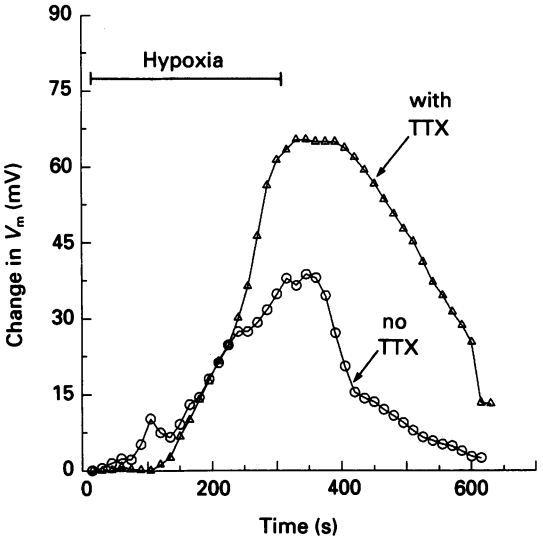

Fig. 7. Plots showing change in membrane potential, V_m, as a function of time during hypoxia and reoxygenation with and without TTX. Time 0 = start of hypoxia, 300 s = end of hypoxia or start of reoxygenation. Note that the membrane potential change in the presence of TTX was not reduced and even exaggerated. V_m was −71 mV just before hypoxia was instituted, and, in the presence of TTX, there was an overshoot in V_m. Note repolarization with reoxygenation in both conditions.

other experiments we attempted to produce in newborn neurons the same magnitude of change in V_m that we observed in adult neurons ($n = 5$). To depolarize the newborn cells by 23–27 mV required a hypoxic exposure of 15–30 min, i.e. about 3× longer than the adult exposure time. Despite this prolonged period of hypoxia, neonatal cells continued to fire when evoked, though with action potentials of smaller amplitudes. With this prolonged exposure to hypoxia, there was no consistent change in R_N in neonatal cells.

The depolarization observed at 21 and 28 days was much larger than that observed in neurons of younger rats (Fig. 2, lower panel). The average maximal change in V_m at 21 days of age was 25·5 ± 9·6 mV and was significantly different from that of younger neurons ($P < 0{\cdot}01$). It was also significantly different from that of 28-day-old and adult neurons ($P < 0{\cdot}01$). There was no significant difference in the mean maximal depolarization between 28-day-old and adult neurons.

Pre- or postsynaptic phenomenon

Since synaptic potentials increased during hypoxia, we asked whether the observed depolarization is a result of pre- or a postsynaptic phenomenon. In separate experiments, we studied the effects of hypoxia in the presence of TTX (0·3–1 μM;

$n = 3$), Ca^{2+}-free Ringer with $CoCl_2$ ($n = 6$) or low Ca^{2+} (0·5 mM)/high Mg^{2+} (3·5 mM) ($n = 3$) containing solutions. Action potentials could not be generated with injected current post-TTX and synaptic membrane noise was markedly reduced with Ca^{2+}-free (with $CoCl_2$) or low Ca^{2+}/high Mg^{2+} solutions before hypoxic exposure was

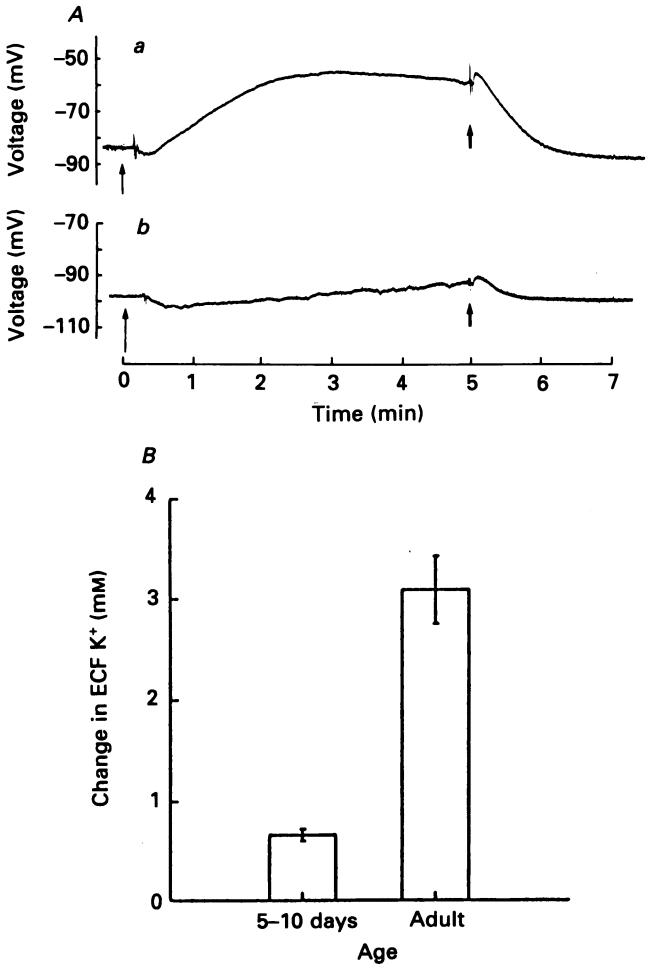

Fig. 8. *A*, effect of exposure to low oxygen on tissue K^+ activity. *A a*, in adult brain stem, ECF K^+ increased during 5 min of hypoxia from 2·8 mM (−93·0 mV) to a maximum of ~ 7 mM (−64·0 mV). Typically, most of the increase occurred between 1 and 3·5 min, levelling off or even slightly dropping subsequently. *A b*, change in ECF K^+ of newborn tissue was minimal from 2·5 mM (−96·0 mV) to a maximum of ~ 2·9 mM (−92·0 mV). For both *A a* and *b*, the first arrow indicates the start of hypoxia and the second indicates the end of exposure. *B*, average change in ECF K^+, compared to control, measured in adult and newborn tissue after 5 min of hypoxia.

attempted. The depolarization trajectory was either unchanged or sometimes exaggerated as compared to that obtained in normal Ringer solution containing no TTX (Fig. 7). At no time was the depolarization attenuated post-TTX. Similarly, Ca^{2+}-free Ringer with $CoCl_2$ or low Ca^{2+}/high Mg^{2+} solutions did not influence the

magnitude of the depolarization. Thus, these data strongly suggest that the depolarization is not secondary to synaptic interactions.

K^+ channel blockers

Because of the importance of K^+ in determining membrane potential and the V_m changes during hypoxia (Fujiwara *et al.* 1987), we used TEA ($n = 4$) before and through the hypoxic experimental run. As was anticipated, neurons depolarized and actions potentials widened markedly on exposure to TEA before hypoxia was instituted. With hypoxia, the depolarization increased further and the maximum change in potential was greater with TEA than without. Experiments using apamin ($n = 2$) in small concentrations (50–100 pM) to block Ca^{2+}-activated K^+ channels (Romey, Duval, Vincent & Lazdunski, 1982) did not change V_m trajectory during hypoxia.

Intracellular EGTA

Ionophoresing EGTA by hyperpolarization ($n = 6$) did not prevent the depolarization induced by hypoxia in 4-week-old and adult rats. Similar depolarizing trajectories were obtained after EGTA ionophoresis of 15 min.

Hypoxia and extracellular K^+

Extracellular fluid K^+ ion activity was measured in neonatal and adult brain tissue. During each experiment, microelectrodes were placed at the same depth in the neonate and adult tissue. Twelve experiments were performed on newborn (5- to 7-day-old) and adult slices using the Corning LIX. After a latency of about 30 s, adult ECF K^+ concentration started to increase. This increase was most dramatic between 1 and 3 min into hypoxia and generally increased by about 3–3·5 mM after 4 min into hypoxia at which time K^+ levels levelled off (Fig. 8*A a*). In contrast, the increase in ECF K^+ in the neonate was minimal and averaged about 10–20 % of the adult values (Fig. 8*A b* and *B*).

Since the Corning LIX is very sensitive to choline/acetylcholine, we ascertained the difference between newborns and adults in three additional experiments using the Fluka LIX which is much less sensitive to acetylcholine and more selective for K^+ than the Corning LIX (see Methods). Similar differences were obtained between the newborn and adult slices. Adult hypoglossal areas had an increase of about 3–4 mM by 5 min into hypoxia while newborn hypoglossal areas had little increase (0·2 mM).

Strophanthidin application

Since hypoxia may deplete ATP and inhibit the Na^+–K^+ pump and since this inhibition may be at the basis of the electrophysiological changes we observed, in separate experiments we applied strophanthidin, a Na^+–K^+ pump blocker, to study its effects on membrane potential and input resistance. Strophanthidin induced a depolarization of all adult neurons tested ($n = 6$). The depolarization was gradual and R_N was measured at discrete times during the course of depolarization. At a time when V_m was reduced by 20–30 mV (a reduction commensurate with that observed

during hypoxia in the adult neurons), R_N had decreased in five out of six cells (Fig. 9) and the average decrease in R_N was about 40%.

DISCUSSION

This is the first study that we known of which examines the neuronal basis for the electro-responsiveness of brain stem neurons to hypoxia with development. Our data

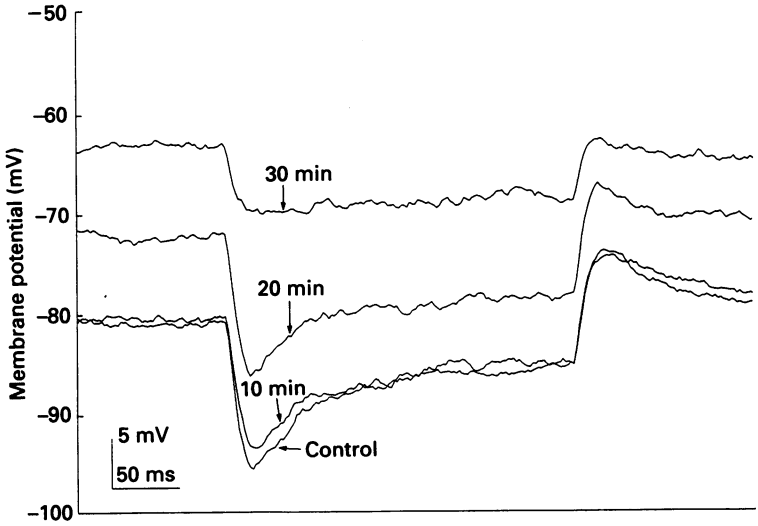

Fig. 9. Effect of strophanthidin (50 μM) on R_N. Note the drop in voltage change for the same hyperpolarizing step as a function of time during strophanthidin application.

show that major electrophysiological changes occur during O_2 deprivation after 21–28 days but not in the first 2–3 weeks of age, suggesting that fundamental differences exist between adult and neonatal neurons in their responsiveness to hypoxia. Adult hypoglossal neurons depolarize and increase membrane input resistance in response to O_2 deprivation. In contrast, newborn neurons are more tolerant of hypoxia, and maintain well neuronal excitability, repetitive firing properties and ECF K^+ in the face of decreased oxygen availability. These functions are also preserved over 15 min of hypoxia, i.e. 3 times longer than the hypoxic period that produces depolarization blockade in many adult neurons. A recently published report suggests that these changes may be a general phenomenon in the CNS. Cortical CA1 neurons from young rats show less hypoxia-induced changes in V_m and R_N as compared to adult neurons (Cherubini, Ben-Ari & Krnjevic, 1989).

Although the magnitude of change in V_m is clearly different between age groups, there was considerable variability in the magnitude of individual values. This variability can be ascribed to two factors: (1) unlike most mammals, rats show neurogenesis postnatally in parts of the central nervous system such as the cerebellum and the brain stem. Neuronal proliferation takes place up till about 7–10

days of age in the brain stem (Fish & Winick, 1969). This may increase variability in our data since the 'age' of the neurons at the time of study can vary, (2) since neurons were recorded at varying depths, both baseline P_{O_2} and the change in P_{O_2} with hypoxia could have varied from neuron to neuron.

Neuronal excitability and mechanisms during hypoxia

Although the mechanism underlying the change in neuronal excitability during hypoxia is incompletely understood, the increase in adult ECF K^+ undoubtedly plays a major role in enhancing excitability. The likely source of increased ECF K^+ is from the intracellular compartment. It has been suggested that increased outward flux of K^+ from neurons may account for the increased ECF K^+ during increased neuronal activity (Baylor & Nicholls, 1969; Orkland, 1980; Connors, Ransom, Kunis & Gutnick, 1982; Poolos, Mauk & Kocsis, 1987) as in our adult hypoglossal neurons during hypoxia. However, neurons may not be the only K^+ source. Although glial cells are well known to take up K^+ and are much more numerous in the adult than in the newborn, their function during O_2 deprivation is not well understood, especially when ECF K^+ increases.

Although it is likely that an increased outward flux of K^+ occurs during hypoxia and accounts for the increased ECF K^+, changes in ECF size may determine the magnitude of change in K^+. This is especially important since the size of the ECF is larger in newborn than in adult brains (Van Harreveld, 1972). Thus, for the same K^+ flux, the magnitude of the increase in K^+ could be less in newborn brains. Furthermore, electrical activity, as we observed in adult hypoglossal neurons during hypoxia, decreases the size of the ECF and this decrease may enhance the magnitude of K^+ change observed in the adult slice (Ransom, Yamate & Connors, 1985). However, this activity-dependent shrinkage may be relatively small (10–15%) even with K^+ concentrations as high as 15–20 mM (Ransom *et al.* 1985). Thus, we believe that changes in ECF are important but probably do not account for a 5-fold difference between newborn and adult K_o^+.

Previous *in vivo* experiments have shown that there are three phases in the time course of K_o^+ in the adult brain and that the maximum level reaches is an order of magnitude higher (Hansen, 1985) than levels reached in this study. We have not quantified the trajectory of K_o^+ in our experiments, but is clear from Fig. 8 that we have three stages that are similar to those previously described (Hansen, 1985). Our studies have not shown, however, the excessive amounts of K_o^+. Clearly, there are a number of possible explanations including differences between ischaemia and hypoxia, the effect of the circulatory system, brain regional differences, grey and white matter differences and the level of O_2 deprivation.

In addition to ionic changes, neurochemicals may also play an important role in determining the hypoxic response. Our experiments using TTX, or high Mg^{2+}/low Ca^{2+}, and zero calcium (with $CoCl_2$) perfusion solutions suggested that Ca^{2+}-dependent synaptic processes play, if anything, only a minor role in the hypoxic response. However, these experiments do not rule out a role for neuromodulators. For instance, glutamate release in response to hypoxia determines the response of hippocampal CA3 neurons to hypoxia (Ben-Ari & Lazdunski, 1989). Thus the role of neuromodulators in the brain stem response remains an open question.

Our data in this report and our previous data on vagal motoneurons (Donnelly & Haddad, 1989) show that adult hypoglossal and vagal brain stem neurons depolarize in response to hypoxia. In contrast, cortical hippocampal CA1 neurons in response to hypoxia depolarize slightly for 5–10 s and this is followed by a prolonged hyperpolarization (Fujiwara *et al.* 1987; Leblond & Krnjevic, 1989). The changes in membrane resistance are also different. Adult hypoglossal neurons show increased input resistance in response to hypoxia, in contrast to vagal motoneurons and CA1 neurons which decrease input resistance (Donnelly & Haddad, 1989; Leblond & Krnjevic, 1989). These data demonstrate that various groups of mammalian CNS neurons behave differently in response to hypoxia. They also suggest that, although K_o^+ is an important determinant of V_m, it cannot fully explain response differences between the hippocampus and brain stem neurons since an increase in K_o^+, albeit small, has also been reported in the hippocampus during hypoxia (Fujiwara *et al.* 1987). This is supported by preliminary unpublished observations from our laboratory showing that hypoglossal neurons do not depolarize nearly as much (15–20 mV) when bathed with K^+ of 10–11 mM as when exposed to hypoxia which, on average, increases K^+ by about 3–4 mM. It is likely, therefore, that the inherent membrane and cellular properties and the presence or predominance of certain ion channels play an important role in determining the electro-responsiveness of brain stem or cortical neurons to hypoxia.

ATP depletion and neuronal excitability

Recent data (Kass & Lipton, 1989) describing the effect of anoxia on ATP levels in slices taken from young and adult rats shed some light on the mechanisms underlying the ability (or lack thereof) of neurons to maintain ionic gradients across neuronal membranes. Although the young rats in their study were older (age 4–6 weeks) than our young rats, they nevertheless showed a lower rate of drop in ATP levels than the adult rats. Hence, similar or even lower rates of decrease in ATP levels would probably be expected to occur in neonates. This difference in ATP levels during hypoxia between the young and the older animals (Kass & Lipton, 1989) could serve as an explanation for the difference in the degree of maintenance of ionic gradients across neuronal membranes. Based on this study, it is possible that the Na^+–K^+ pump, which is an important basal energy-requiring process in neurons, fails in the adult neuron but is much less affected in the newborn. This would be consistent with a sharp rise in K_o^+ in the adult tissue and the small increase in the neonate, as we have shown in our studies.

One of the important features of our studies is that we recorded intracellularly from both newborns and adult hypoglossal neurons and determined the changes in passive properties. R_N increased in the adult hypoglossal cell and remained constant in the neonate. The increase in R_N in the adult was only partially blocked by preventing depolarization and cannot be considered as solely secondary to the change in V_m. This increase in R_N is not consistent with the idea that the sole basis for the ionic movements across neuronal membranes is related to the failure of the Na^+–K^+ pump. To test this hypothesis, we used strophanthidin and demonstrated that although neurons depolarize when Na^+–K^+ pump is blocked, membrane input resistance decreases rather than increases, as our hypoxia results showed. Hence, the

increase in R_N in the adult hypoglossal neuron during hypoxia is the result of another direct postsynaptic process, that of an interruption of a current(s) which is present during baseline recordings but absent during hypoxia.

Recent data have shown that Ca^{2+} plays an important role in neuronal excitability during anoxic brain damage (Siesjo & Bengtsson, 1989). Our data obtained with intracellular EGTA and those of others (Leblond & Krnjevic, 1989; Krnjevic & Leblond, 1989) do not lend support to the Ca^{2+} hypothesis under conditions of our experimental protocols. Furthermore, it is unlikely that Ca^{2+} entry is responsible for the large depolarization seen in the adult hypoglossal neurons, especially since $CoCl_2$ did not change the magnitude of depolarization seen with hypoxia. However, we agree with Leblond and Krnjevic that it is premature to dismiss the Ca^{2+} hypothesis since direct Ca_i^{2+} measurements during O_2 deprivation have not been made and an increase in Ca_i^{2+} from intracellular stores (Siesjo & Bengtsson, 1989; Blaustein, 1988) cannot be ruled out at present.

Upper airway muscle function

Since upper airway muscle function is a major determinant for the genesis and relief of obstructive sleep apnoea (Kuna & Remmers, 1985), an understanding of the mechanisms underlying recruitment of the extrinsic muscles of the tongue (e.g. genioglossus muscle) becomes of paramount importance. Obstructive sleep apnoea may expose hypoglossal neurons to acute hypoxia. Under these conditions, the increase in adult hypoglossal excitability may serve a useful function. Enhanced excitability is useful for recruiting or increasing the tension of the genioglossus muscle fibres for the same respiratory drive. However, if hypoxia continues, neurons may reach a state of depolarization blockade which would be tantamount to functionally removing hypoglossal neurons and enhancing the likelihood of continued airway obstruction. The benefits and liabilities of enhanced excitability are significantly less in the newborn. If hypoxia is prolonged, the likelihood for resuscitation is much better in the newborn than in the adult.

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