Developmental Changes in Membrane Properties of Chemoreceptor Afferent Neurons of the Rat Petrosal Ganglia

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Donnelly, David F. Developmental changes in membrane properties of chemoreceptor afferent neurons of the rat petrosal ganglia. J. Neurophysiol. 82: 209–215, 1999. Carotid body chemoreceptors increase their responsiveness to hypoxia in the postnatal period, but the mechanism for this increase is unresolved. The purpose of the present study was to examine developmental changes in cellular characteristics of chemoreceptor afferent neurons in the petrosal ganglia with the underlying hypothesis that developmental changes occur and may account for the developmental increase in chemoreceptor responsiveness. Chemoreceptor complexes (carotid body, sinus nerve, glossopharyngeal nerve, and petro nasal ganglia) were harvested from rats, aged 3–40 days, and intracellular recordings were obtained from petrosal ganglion neurons using sharp electrode impalement. All chemoreceptor neurons across ages were C fibers with conduction velocities <1 m/s and generated repetitive action potentials with depolarization. Resting membrane potential was $-61.3 \pm 0.9$ (SE) mV ($n = 78$) and input resistance was $108 \pm 6$ MΩ and did not significantly change with age. Cell capacitance was $32.4 \pm 1.7$ pF and did not change with age. Rheobase averaged $0.21 \pm 0.02$ nA and slightly increased with age. Action potentials were followed by an afterhyperpolarization of $12.4 \pm 0.6$ mV and time constant $6.9 \pm 0.5$ ms; only the time constant decreased with age. These results, obtained in rat, demonstrate electrophysiologic characteristics which differ substantially from that previously described in cat chemoreceptor neurons. In general developmental changes in cell characteristics are small and are unlikely to account for the developmental increase in chemoreceptor responsiveness with age.

INTRODUCTION

Carotid body chemoreceptors transduce a decrease in arterial PO$_2$ into an increase in sinus nerve afferent activity, which in turn stimulates respiration. This reflex is weak at birth, resulting in the generation of few action potentials, even during strong stimulation (Blanco et al. 1984), and the magnitude of the response matures during the first few weeks after birth (Blanco et al. 1984; Kholwadwala and Donnelly 1992). Several investigators have suggested that the maturation process primarily occurs at the level of the glomus cell—a secretory cell that is presynaptic to the afferent nerve terminals and that responds to hypoxia by increasing intracellular calcium and enhancing catecholamine secretion (Urena et al. 1994). In support of this contention, the magnitude of the anoxia-induced calcium transient as well as the level of catecholamine secretion has been shown to increase in the postnatal period (Donnelly and Doyle 1994; Sterni et al. 1995).

In contrast to the developmental data available on glomus cell function in this period, no datum is currently available on maturational changes in the characteristics of the primary afferent neuron. However, changes in afferent neuron characteristics may potentially play a critical role in determining the level of spiking activity for a given stimulus. For instance, in one model of hypoxia transduction, the afferent spike is initiated by the summation of excitatory events at the nerve terminals (Hayashida et al. 1980). From this model, it is obvious that a change in excitability (e.g., threshold, input resistance) could greatly affect the translation of these excitatory synaptic events into the output train.

The purpose of the present study was to examine developmental changes in membrane properties of chemoreceptor afferent neurons with the underlying assumption that the soma and nerve terminals share similar membrane characteristics. Thus observations at the soma may be useful in understanding processes that take place in the nerve terminal. Such similarity is well established for baroreceptor afferent fibers that possess stretch activated channels in the soma and peripheral processes (Kraske et al. 1998). The hypothesis of the present study was that developmental changes occur and may explain the developmental increase in chemoreceptor responsiveness.

METHODS

Experimental preparation

Both carotid body/sinus nerve/petrosal ganglia (chemoreceptor complex) were isolated from 28 rats, aged 3–40 days, using a methodology adapted from the original work of Belmonte and Gallego who isolated the chemoreceptor complex from adult cats (Belmonte and Gallego 1983). For the isolation, rats were anesthetized by placement in a closed chamber in which the atmosphere was saturated with methoxyflurane vapor. After deep anesthesia as evidenced by an absence of motor movements, outside of respiration, the rats were removed and decapitated. The carotid body was exposed by dissecting medial to the carotid artery and removing the superior cervical ganglia. The petrous bone was split and the central portion of the vagal medulla to the carotid body/sinus nerve/petrosal ganglia. Both carotid body/sinus nerve/petrosal ganglia (chemoreceptor complex) were isolated from 28 rats, aged 3–40 days, using a methodology adapted from the original work of Belmonte and Gallego who isolated the chemoreceptor complex from adult cats (Belmonte and Gallego 1983). For the isolation, rats were anesthetized by placement in a closed chamber in which the atmosphere was saturated with methoxyflurane vapor. After deep anesthesia as evidenced by an absence of motor movements, outside of respiration, the rats were removed and decapitated. The carotid body was exposed by dissecting medial to the carotid artery and removing the superior cervical ganglia. The petrous bone was split and the central portion of the vagal and glossopharyngeal nerves were dissected free and reflected over the carotid bifurcation. The bifurcation and associated nerves were cut free and placed in chilled, oxygenated (95% O$_2$:5% CO$_2$) Ringer saline [containing (in mM) 125 NaCl, 3 KCl, 1 NaH$_2$PO$_4$, 2 CaCl$_2$, 1.5 MgSO$_4$, 26 NaHCO$_3$, and 10 glucose].

Using sharp dissection, the area was cleaned with the removal of vagal fibers, carotid arteries, and connective tissue, and the remaining tissue was placed in a dilute solution of collagenase/trypsin (0.1%/0.01%) in oxygenated Ringer saline for 30 min to aid in further cleaning. After exposure to the enzymes, the connective tissue was gently pulled/ cut away using a fine pipette and scalpel blade until the carotid body/sinus

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nerve/glossopharyngeal nerve and petrosal ganglia were easily visible and free of connective tissue. The chemoreceptor complex was transferred to a perfusion chamber mounted on the stage of an inverted microscope, and the preparation was superfused with Ringer saline equilibrated with 21% O₂-5% CO₂ and heated to 32–33°C.

Stimulus electrode

An insulated metal microelectrode (Frederick Haer) with a 5-μ exposed tip was advanced into the carotid body under visual control. While searching for a chemoreceptor unit or during unit characterization, a cathodal electrical stimulus (0.1-ms duration) was delivered through an optically isolated, constant current source (BAK instruments, BSI-1) to evoke an orthodromic action potential. Initial current level was set at 800 μA, which is ~10–20 times higher than the minimal current necessary to evoke an orthodromic action potential.

Intracellular recording

Intracellular recordings from the soma of petrosal neurons were obtained using micropipettes filled with 3 M KCl and connected to an electrometer (IE-210, Warner Instruments) the output of which was filtered from 0 to 2 kHz. Tip resistance was 80–120 MΩ. A chemoreceptor petrosal neuron was identified based on the orthodromic action potential initiated from the stimulus electrode placed in the carotid body. Criteria for an acceptable recording included a stable membrane potential for ≥5 min after cell puncture.

Passive properties

Characterization of passive membrane properties included measurement of resting membrane potential and input resistance. Input resistance was based on the steady-state magnitude of hyperpolarization during application of a 0.1-nA hyperpolarizing current. For measurement of membrane time constant, the charging transient at the start of application of a 0.1-nA hyperpolarizing current was fit to a single order exponential function (pClamp, Axon Instruments). Estimation of membrane capacitance was calculated by dividing the membrane time constant by the input resistance.

Spike properties

All spike properties were based on measurements obtained from orthodromic spikes elicited by current pulses applied to the carotid body. Control of the stimulus pulse and data acquisition were controlled by pClamp software (Axon Instruments) at 10-kHz digitization rate. Spike rise time was the time period for the spike to rise from 10 to 90% of its peak value. Action potential height was the difference between the peak of the action potential waveform and the resting membrane potential. Afterhyperpolarization potential (AHP) was the difference between the resting membrane potential and the maximum hyperpolarization after a spike. AHP time constant was estimated by a best-fit approximation of the post-AHP potential to a single exponential function.

Repetitive spiking properties

Rheobase was measured as the minimal current necessary to evoke at least one action potential. The stimulus current was generally applied for 100 ms and was incremented by 0.1 nA over the range of 0.1–2 nA. In some cases, the stimulus was applied between 0.05 and 1 nA in increments of 0.05 nA. Peak discharge frequency was calculated as the instantaneous frequency between the first and second spikes during application of a 0.8-nA depolarizing stimulus. If no repetitive spike discharge was observed, then it was deemed that the cell was unable to support repetitive spike generation.

Spontaneous action potential and response to cyanide

In seven cells that initially were identified as chemoreceptor afferent fibers based on orthodromic electrical stimulation, the response to brief application was tested. Cyanide (0.5 mM, 2 s) was applied locally to the carotid body through micropipette using positive ejection pressure (Picospritzer, General Value). Cells, which responded by an increase in spontaneous discharge rate, were considered as confirmed chemoreceptor fibers and used as a comparison population for the larger sample.

Data analysis

Each variable was plotted against the age of the rat from which the chemoreceptor complex was harvested. A linear regression against age was calculated as well as the 95% confidence interval for the regression line (Systat). A significant effect of age on the measured variable was established if the regression slope was significantly different from 0 at the 0.05 level. Data also were grouped by age into three age groups: 1–10 days, 11–20 days, and >20 days and analyzed for an effect of age using ANOVA. Post hoc testing was accomplished Student’s t-test.

RESULTS

Conduction velocity

Application of a cathodal electrical stimulus to the carotid body evoked an orthodromic action potential in both the older (Fig. 1) and in the newborn rat (Fig. 2). The latency between the stimulus and evoked spike was 3.53 ± 0.13 (SE) ms for the entire population (n = 78) but significantly increased ~2.5-fold with age (P < 0.001; Fig. 3). This was primarily due to an increase in conduction distance because the estimated conduction velocity for the population was 0.52 ± 0.02 m/s and increased only slightly with age (P < 0.05; Table 1).

Passive properties

RESTING MEMBRANE POTENTIAL AND INPUT RESISTANCE. Resting membrane potential for the entire population was ~61.3 ± 0.9 mV (n = 78); however, there was considerable variability among individual cells. This seemed in part due to a variable response to electrode impalement that usually caused a brief depolarization followed by a hyperpolarization beyond ~80 mV, followed by a slow depolarization and stabilization. ANOVA analysis of resting membrane potential showed no difference among the three age groups (Table 1). Input resistance, as measured with a hyperpolarization step of 0.1 nA averaged 108 ± 6 MΩ, and, like the resting membrane potential, did not change significantly with age (Table 1).

MEMBRANE TIME CONSTANT AND CAPACITANCE. Membrane time constant was estimated by fitting the charging transient for a 0.1-nA hyperpolarization pulse to a single-order exponential. Average membrane time constant was 3.1 ± 0.2 ms for the entire population. Time constant significantly decreased with age (P < 0.001, Fig. 4, Table 1) with approximately a 50% decrease over the period of 3–40 days. Dividing each time constant by the input resistance yielded an estimate of membrane capacitance that averaged 32.3 ± 1.7 pF for the entire population. Age had no significant effect on capacitance among the three age groups (Table 1).
Active properties

SPIKE PROPERTIES. Spike height for the population averaged 58.4 ± 1.3 mV and was not significantly different among the three age groups (Table 1). However, the spike rise time, which averaged 0.44 ± 0.2 ms for the entire population, did significantly decrease with age ($P < 0.05$; Table 1). The spike was followed by an AHP that averaged 12.4 ± 0.6 mV and had an average time constant of ~6.9 ± 0.5 ms (Figs. 1 and 2). The AHP amplitude was not significantly different among age groups (Fig. 4, Table 1), but the time constant shortened significantly ($P < 0.05$) by ~40% during the developmental time period (Fig. 4, Table 1). Differentiation of the spike-wave failed, in all instances, to demonstrate a “hump” on the falling phase (Figs. 1 and 2). A hump on the falling phase is characteristic of many chemoreceptor neurons from the adult cat and suggests the presence of a significant calcium current in cat neurons (Belmonte and Gallego 1983).

RHEOBASE AND REPETITIVE DISCHARGE ACTIVITY. The minimum injected current needed to elicit an action potential averaged 0.21 ± 0.02 nA for the entire population and slightly, but significantly ($P < 0.05$), increased with age (Fig. 4, Table 1). Injections of greater amounts of current evoked multiple action potentials in every cell tested (Figs. 1 and 2), and, during injection of 0.8 nA the average spiking rate was 139 ± 5 Hz. Age had no significant effect on the spiking rates observed during this current injection (Table 1).

RESPONSE TO CYANIDE. In the above sample, identification of purported chemoreceptor afferent neurons was based on the ability to orthodromically activate the afferent from within the carotid body. To test the chemoresponsiveness of these cells, cyanide (0.5 mM) was “spritzed” on the carotid body in seven cases. In five of these cells, an increase in spontaneous spiking activity was observed readily after cyanide application (Fig. 5). Analysis of the spontaneous and evoked action potentials for these five cells were consistent with the averages obtained on the initial sample and the spontaneous action potentials were identical to those obtained using orthodromic electrical stimulation (Fig. 5).

DISCUSSION

This is the first study to characterize the electrophysiological properties of petrosal chemoreceptor neurons of the rat and to
examine developmental changes in the neuronal characteristics. Previous studies examined chemoreceptor neurons of the adult cat, and it is now apparent that there is a marked species difference between rat and cat in a number of variables. These issues will be considered in the following text, but it is first worth considering the issue of developmental changes in chemoreceptor responsiveness.

Previous results from our laboratory and others have demonstrated a large developmental change in chemoreceptor responsiveness in the newborn period. Fetal and newborn chemoreceptors generate significantly fewer spikes in response to a strong hypoxic stimulus than do chemoreceptors from older animals (Blanco et al. 1984; Kholwadwala and Donnelly 1992). The enhanced spiking activity may be due to a greater level of stimulation of the nerve terminals or, potentially, due to changes in the sensitivity of the nerve terminals to stimulation. Regarding the first possibility, considerable data have been developed showing developmental changes in the characteristics of the presynaptic element, the glomus cell. First, the magnitude of the hypoxia-induced increase in intracellular calcium increases with development (Sterni et al. 1995), and this is correlated with an increase in the magnitude of hypoxia-induced catecholamine release from these cells (Donnelly and Doyle 1994). On the other hand, it is currently unclear whether the number of observations is given in parentheses. A significant change of the variable with development is indicated in the right column. AP, action potential; AHP, after hyperpolarization. NS, nonsignificant. Parenthetical numbers indicate a significant change with age.

<table>
<thead>
<tr>
<th>Variable</th>
<th>1–10 Days</th>
<th>11–20 Days</th>
<th>20–35 Days</th>
<th>ANOVA</th>
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<tr>
<td>V&lt;sub&gt;m&lt;/sub&gt;, mV</td>
<td>−59.5 ± 1.4</td>
<td>−61.2 ± 1.7</td>
<td>−63.5 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>R&lt;sub&gt;n&lt;/sub&gt;, MΩ</td>
<td>118 ± 10</td>
<td>101 ± 6</td>
<td>103 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>Conduction velocity, m/s</td>
<td>0.46 ± 0.03</td>
<td>0.53 ± 0.03</td>
<td>0.56 ± 0.04</td>
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<td>Time constant, ms</td>
<td>3.89 ± 0.30</td>
<td>3.08 ± 0.24</td>
<td>2.34 ± 0.21</td>
<td>↓ (P &lt; 0.001)</td>
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<td>Capacitance, pF</td>
<td>36.3 ± 3.7</td>
<td>31.4 ± 2.4</td>
<td>28.5 ± 2.7</td>
<td>NS</td>
</tr>
<tr>
<td>AP height, mV</td>
<td>61.8 ± 1.7</td>
<td>52.8 ± 2.8</td>
<td>60.1 ± 2.5</td>
<td>NS</td>
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<td>Rise time, ms</td>
<td>0.49 ± 0.04</td>
<td>0.53 ± 0.03</td>
<td>0.40 ± 0.03</td>
<td>↓ (P &lt; 0.05)</td>
</tr>
<tr>
<td>AHP magnitude, mV</td>
<td>12.0 ± 0.9</td>
<td>13.7 ± 1.0</td>
<td>10.5 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>AHP time constant, ms</td>
<td>8.0 ± 0.7</td>
<td>6.6 ± 0.7</td>
<td>5.4 ± 1.0</td>
<td>↑ (P &lt; 0.05)</td>
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<td>Rheobase, nA</td>
<td>0.18 ± 0.02</td>
<td>0.22 ± 0.03</td>
<td>0.28 ± 0.04</td>
<td>↑ (P &lt; 0.05)</td>
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<tr>
<td>Peak frequency, Hz</td>
<td>127 ± 7</td>
<td>137 ± 8</td>
<td>137 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>Number of observations</td>
<td>26</td>
<td>23</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Number of rats</td>
<td>6</td>
<td>9</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 3. Latency to orthodromic spike and conduction velocity as a function of age. A: distribution of latencies between the stimulus artifact and arrival of the orthodromic spike. Latency significantly increased with age. B: estimated conduction velocity based on the latency to the orthodromic spike and estimate of conduction distance. All conduction velocities were <1 m/s and did not significantly change with age. Each panel shows the regression line and 95% confidence limit for the regression line.

FIG. 4. Changes in cell characteristics with age. A: distribution of membrane time constants as measured during application of a −0.1-nA current pulse. Time constant significantly decreased with age. B: distribution of values for the magnitude of the postspike AHP. AHP magnitude did not significantly change with age. C: distribution of values for the time constant for recovery from the AHP. This time constant significantly decreased with age. D: distribution of current values that evoke ≥1 action potential. Rheobase slightly, but significantly, increased with age.
glomus cell secretion of catecholamines causes the increase in afferent nerve activity. Under several experimental conditions, there is a dissociation between the magnitude of catecholamine secretion and magnitude of nerve activity (Buerk et al. 1997; Donnelly 1996b; Iturriaga et al. 1996), suggesting that hypoxia transduction may, at least in part, be due to release of other transmitters such as substance P (Prabhakar et al. 1993) or acetylcholine (Fitzgerald and Shirahata 1994). Alternatively, the nerve terminal itself may have an endogenous sensitivity to hypoxia (Mitchell et al. 1972).

Regardless of whether the nerve terminals are a site of hypoxia transduction or not, the nerve terminals represent a potential site for maturational changes in organ function. Although direct electrophysiologic study of the nerve terminals would be the preferred experiment, such recordings are extremely difficult due to their small size (McDonald 1981), and, instead, recordings from the soma were used as a proxy for the membrane characteristics. From this standpoint, the developmental changes are relatively minor and are unlikely to account for the developmental increase in chemoreceptor responsiveness. The lack of significant changes in resting potential or input resistance suggests that no major changes occur in the passive characteristics of these neurons. Rheobase, which may have been expected to increase with development, actually decreased slightly with development. Furthermore peak repetitive firing rates for chemoreceptor afferents are considerably faster than that observed using physiological stimulation and do not change with age. Thus the low spiking rates in the newborn period are not due to a limitation of the afferent neuron in supporting repetitive action potential generation. Given that the membrane characteristics are not different with development, a better explanation for the maturational increase in activity is the enhancement in glomus cell stimulus/secretion coupling with development (Donnelly and Doyle 1994) or the maturational increase in the number of axonal terminals per parent axon with a corresponding increase in the number of spike generating sites (Kondo 1976). In addition, it is well established that survival of the chemoreceptor afferent fibers as well as transmitter phenotype is dependent on oxygen and depolarizing stimuli in the fetal and newborn period (Erickson et al. 1998; Hertzberg et al. 1995). How these phenotypic changes are related to electrophysiologic characteristics currently is unexplored and may form a useful focus for future studies.

FIG. 5. Response to cyanide. Intracellular recording from the soma of a 14-day petrosal chemoreceptor neuron before (A) and after (B) application of cyanide to the surface of the carotid body. Somal electrophysiologic characteristics were consistent with that obtained from purported chemoreceptor afferent neurons identified by orthodromic electrical stimulation (Table 1). $V_m = 59 \text{ mV}$; AHP $\approx 8 \text{ mV}$; AHP time constant $\approx 7 \text{ ms}$.
Identification of purported chemoreceptor neurons was based on the ability to orthodromically generate an afferent spike and the response to chemostimulating agents was not routinely tested. Thus some nonchemoreceptive neurons may have contributed to the sampled population, perhaps due to electrical stimulation of C-fiber axons that pass over the carotid body. However, it seems likely that the level of contamination is small. C-fiber axons of passage are difficult to stimulate electrically and require very high voltages or stimulus currents. For instance, the threshold for C-fiber activation using direct sciatic nerve stimulation is $>100$ V (Traub and Mendell 1988), which is beyond the voltage compliance of the stimulus isolator used in the present study. In addition, the majority (5/7) of cells that were tested for chemosensitivity showed spontaneous spiking activity and an increase in activity after cyanide application. Spike characteristics for these cells were consistent with the larger population identified by orthodromic electrical stimulation.

Species dependence

In contrast to previous studies on cat carotid body (Belmonte and Gallego 1983), virtually all rat chemoreceptor afferents were C fibers—a result generally consistent with observation that $>86\%$ of rat sinus nerve fibers are unmylelinated (McDonald 1983). Estimated somal capacitance was also small at 32 pF. This characterization is consistent with the histological assessment of cell characteristics after retrograde tracer application to the rat carotid body, which primarily labeled the smallest cells of the petrosal ganglia (Finley et al. 1992). In contrast to the rat, the vast majority of cat chemoreceptor petrosal neurons were reported to have conduction velocities $>10$ m/s (Belmonte and Gallego 1983), and somal capacitance approximately three times larger than the rat (Belmonte and Gallego 1983).

A second major difference between cat and rat appears to be characteristics of action potential and ability to generate multiple spikes. Rheobase for the rat cells averaged $\sim$0.2 nA with many cells initiating spikes at lower levels of current injection. For cat cells, the comparable value is $\sim$1 nA (Belmonte et al. 1988; Gallego et al. 1987). Cat cells also fail to generate multiple spikes at higher levels of current injection, and, instead, generate only one to two spikes (Belmonte and Gallego 1983). In contrast, all of the rat chemoreceptor cells generated multiple spikes during strong depolarizations. The ionic basis for this species difference is unclear since action potentials of both species are followed by AHPs of similar magnitude (Belmonte and Gallego 1983). However, the duration of the AHP is almost 10 times longer in the cat cells, perhaps impairing the ability to initiate new spikes before depolarization-induced inactivation of the Na$^+$ current. The long AHP in the cat cells appears to be caused by a pronounced calcium current during the action potential and activation of calcium-dependent K$^+$ currents (Belmonte and Gallego 1983). This calcium current may not be shared with rat cells because no hump on the falling phase of the action potential was observed in any recordings from the rat.

Given these differences, it is perhaps surprising that the afferent spike trains from rat and cat chemoreceptors are similar. In both cases, the afferent spike train approximates a Poisson random process with some deviation due to the refractory period and a tendency of some fibers to discharge in doublets (Donnelly 1996a; Eyzaguirre and Koyano 1965). Although the postspike refractory period in the rat ($\sim$8 ms) is similar to the observed duration of somal AHP, this is not true of the cat, which has a much longer AHP (Belmonte and Gallego 1983). It is currently unclear why the long AHP is not manifest in the interspike interval histogram of cat chemoreceptors, but the result suggests that the cat nerve terminals may have different membrane characteristics than the soma.

In summary, the present results demonstrate that electrophysiologic characteristics of chemoreceptor rat petrosal neurons are relatively stable in the postnatal period and developmental changes in the cellular characteristics are unlikely to account for the postnatal increase in chemoreceptor responsiveness. However, the results also demonstrate that rat chemoreceptor neurons differ substantially from those previously reported from adult cat, particularly in conduction velocity, rheobase and ability to support multiple action potential generation. It is hoped that knowledge of the excitable properties of the afferent fibers will ultimately allow us to model the spike generation process of the arterial chemoreceptors.

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