Development of Electrophysiological and Morphological Diversity in Autonomic Neurons

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Anderson, Rebecca L., Phillip Jobling, and Ian L. Gibbins. Development of electrophysiological and morphological diversity in autonomic neurons. J Neurophysiol 86: 1237–1251, 2001. The generation of neuronal diversity requires the coordinated development of differential patterns of ion channel expression along with characteristic differences in dendritic geometry, but the relations between these phenotypic features are not well known. We have used a combination of intracellular recordings, morphological analysis of dye-filled neurons, and stereological analysis of immunohistochemically labeled sections to investigate the development of characteristic electrical and morphological properties of functionally distinct populations of sympathetic neurons that project from the celiac ganglion to the splanchnic vasculature or the gastrointestinal tract of guinea pigs. At early fetal stages, neurons were significantly more depolarized at rest compared with neurons at later stages, and they generally fired only a single action potential. By mid fetal stages, rapidly and slowly adapting neurons could be distinguished with a topographic distribution matching that found in adult ganglia. Most rapidly adapting neurons (phasic neurons) at this age had a long afterhyperpolarization (LAH) characteristic of mature vasomotor neurons and were preferentially located in the lateral poles of the ganglion, where most neurons contained neuropeptide Y. Most early and mid fetal neurons showed a weak M current, which was later expressed only by rapidly-adapting and LAH neurons. Two different A currents were present in a subset of early fetal neurons and may indicate neurons destined to develop a slowly adapting phenotype (tonic neurons). The size of neuronal cell bodies increased at a similar rate throughout development regardless of their electrical or neurochemical phenotype or their topographical location. In contrast, the rate of dendritic growth of neurons in medial regions of the ganglion was significantly higher than that of neurons in lateral regions. The apparent cell capacitance was highly correlated with the surface area of the soma but not the dendritic tree of the developing neurons. These results demonstrate that the well-defined functional populations of neurons in the celiac ganglion develop their characteristic electrophysiological and morphological properties during early fetal stages of development. This is after the neuronal populations can be recognized by their neurochemical and topographical characteristics but long before the neurons have finished growing. Our data provide strong circumstantial evidence that the development of the full phenotype of different functional classes of autonomic final motor neurons is a multi-step process likely to involve a regulated sequence of trophic interactions.

INTRODUCTION

Neurons vary widely in their expression of ion channels, especially voltage-dependent $K^+$ channels and $Ca^{2+}$-dependent $K^+$ channels. These channels are a fundamental determinant of the firing properties of neurons and their responses to synaptic inputs (Hille 1992). Integration of convergent synaptic inputs also is dependent on interactions between the distribution of ion channels and the dendritic morphology of the neurons. The generation of neuronal diversity clearly requires the coordinated development of these features (Dryer 1994, 1998; Ribera and Spitzer 1992). However, the relationship between differential ion channel expression and dendritic morphology during the development of mature neuronal phenotypes is not well known.

Autonomic pathways comprise one of the primary motor outputs of the nervous system and contain more final motor neurons than any other pathway. In humans, there are more than 10 million final motor neurons in sympathetic pathways alone (Gibbins 1990). Compared with somatic final motor neurons, autonomic neurons show a great diversity of phenotypic characteristics, such as their neuropeptide content, electrical properties, morphology, and synaptic connectivity. In addition, autonomic neurons found in specific functional pathways often express precise combinations of these phenotypic characteristics (Adams and Harper 1995; Andrews et al. 1996; Chiba and Tanaka 1998; Dryer 1994; Gibbins 1995; Jobling and Gibbins 1999; Morris et al. 1997–1999; Smith 1994). The celiac ganglion of guinea pigs provides a striking example of this phenomenon. Here, vasomotor neurons can be distinguished from neurons projecting to the enteric plexuses by their location, the size of their dendritic fields, their neuropeptide content, the potassium channels they express, and the origins and number of their synaptic inputs (Boyd et al. 1996; Cassell and McLachlan 1987; Cassell et al. 1986; Costa and Furness 1984; Davies et al. 1999; Gibbins et al. 1999; Keast et al. 1993; Lindh et al. 1986; Macrae et al. 1986; McLachlan and Meckler 1989; Meckler and McLachlan 1988) (Table 1).

Although autonomic neurons have been used to study many different aspects of neuronal differentiation (Dryer 1994, 1998; Dryer and Chiappinelli 1985; Hirst and McLachlan 1984; McFarlane and Cooper 1992, 1993; Nerbonne and Gurney 1989; Phelan et al. 1997; Rubin 1985a–c), few studies have examined the development of phenotypic diversity within functionally identified pathways (Cameron and Dryer 2000; Stofer and Horn 1990, 1993). Indeed, most studies of neuronal development have investigated only a single class of neurons. In principle, the generation of different neuronal phenotypes from a common precursor pool could occur by the sequential

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TABLE 1. Phenotypic characteristics of major classes of functionally identified neurons in mature guinea pig coeliac ganglion

<table>
<thead>
<tr>
<th>LAH Neurons</th>
<th>Tonic Neurons</th>
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<tbody>
<tr>
<td>Main target</td>
<td>Blood vessels</td>
</tr>
<tr>
<td>Location in ganglion</td>
<td>Lateral</td>
</tr>
<tr>
<td>Neuropeptide content</td>
<td>NPY</td>
</tr>
<tr>
<td>Dendritic field size</td>
<td>Small</td>
</tr>
<tr>
<td>(I_{\text{A}})</td>
<td>Present</td>
</tr>
<tr>
<td>(I_{\text{M}})</td>
<td>Present</td>
</tr>
<tr>
<td>(I_{\text{N}})</td>
<td>Small, inactive at rest</td>
</tr>
<tr>
<td>Slow (I_{\text{A}})</td>
<td>Absent</td>
</tr>
<tr>
<td>Firing properties</td>
<td>Phasic (rapidly-adapting)</td>
</tr>
<tr>
<td>Peripheral synaptic inputs</td>
<td>Absent</td>
</tr>
</tbody>
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See text for references. LAH, long after hyperpolarization; NPY, neuropeptide Y; Som, somatostatin.

acquisition of characteristics from a basal embryonic phenotype, such that one mature phenotype was derived from another. Alternatively, each mature phenotype could develop directly from a specific subset of precursors. The resolution of this question has been hampered by the difficulty of identifying different pools of precursor neurons prior to their differentiation. In this study, we have tackled this question by taking advantage of the unique organization of the guinea pig celiac ganglion, which allows us to follow the development of phenotypically diverse populations of neurons that innervate distinct target tissues. We have investigated the differentiation of two major phenotypic characteristics, the differential expression of ion channels, and dendritic morphology in neurons whose functional pools can be recognized from an early developmental stage simply on the basis of their location. To do this, we used intracellular electrophysiological recording techniques, combined with dye-filling, multiple-labeling immunohistochemistry, and confocal microscopy. We have found that many of the electrophysiological characteristics of the main functional classes of neurons develop directly from undifferentiated precursors and can be distinguished from each other long before the neurons finish growing. This suggests that the differentiation of these two phenotypic characteristics is likely to be independently regulated.

METHODS

Pregnant guinea pigs, fetuses, neonates (P1–P13) and nonpregnant adults (>240 g; Cavia porcellus, Hartley/IMV strain) were given a lethal dose of sodium pentobarbitone (Nembutal, Boxcott Laboratories, Asquith, Australia; 200 mg/kg ip). Nonpregnant adult guinea pigs used in the stereological analysis of neuropil (see following text) were killed by stunning and exsanguination. Late-stage fetuses also were exsanguinated after removal from their extra-embryonic membranes. Fetuses were then weighed and placed in a balanced salt solution (see following text). All procedures were approved by the Animal Welfare Committee of the Flinders University of South Australia.

Guinea pigs have a relatively long and variable gestation period of between 55 and 75 days (Matsumoto et al. 1993; Wein 1974). Embryogenesis occurs during the first 30 days of gestation, while the remainder of the gestational period involves fetal growth (Scott 1937). Since guinea pigs undergo postpartum estrus within hours of giving birth (Stockard and Papanicolou 1917), the day of birth of the previous litter is also day 0 of the following litter. Fetuses were obtained from pregnant guinea pigs during three arbitrary stages of development as previously described (Anderson et al. 2001): early fetal (F30–F35; weight range 1–7 g, mean 3.4 ± 0.3 (SE) g, \(n = 30\)), mid fetal (F36–F45; weight range 10.2–48.9 g, mean 20.7 ± 1.9 g, \(n = 21\)), and late fetal (F46+; weight range 37–79 g, mean 50.4 ± 7.5 g, \(n = 5\)). The early fetal stage of development in guinea pigs is approximately equivalent to the first postnatal week in rats and mice (Butler and Juurlink 1987). The weight of neonatal guinea pigs (P0–P13) used in this study ranged from 93 to 191 g (mean 126.0 ± 8.4 g, \(n = 13\)), while nonpregnant adults ranged from 240 to 329 g (mean 286.0 ± 18.2 g, \(n = 5\)). Where possible, our data were analyzed using the log of the weight since there was an exponential increase in weight with increasing age.

Electrophysiology

Tissue Preparation. Celiac ganglia, their nerve trunks, and surrounding tissues (aorta, celiac artery and adrenal glands) were removed and placed into a HEPES-buffered balanced salt solution containing (in mM) 146 NaCl, 4.7 KCl, 0.6 MgSO4, 1.6 NaHCO3, 0.13 NaH2PO4, 2.5 CaCl2, 7.8 glucose, and 20 HEPES, buffered to pH 7.3 and gassed with 100% O2. Ganglia were pinned to the base of a recording chamber (Medical System, Greenval, NY) lined with silicon elastomer (Sylgard, Dow Corning, Midland, MI). During electrophysiological recordings, ganglia were maintained at 35°C and superfused with HEPES balanced salt solution at 2.5 ml/min.

At early fetal stages (F30–F35), poorly developed connective tissue did not allow the celiac ganglion to be pinned tightly in the recording chamber. Instead the ganglion was stabilized by leaving it attached to the abdominal aorta, which was slit longitudinally along its dorsal surface and the reflected corners pinned down. In addition, early fetal neurons were small with little cytoplasm (cross-sectional area of soma ~150 \(\mu\)m2) (Anderson et al. 2001). Both of these factors affected the length of time for which impalements could be held as has been reported in other preparations of developing sympathetic ganglia (Dryer and Chiappinelli 1985; Hirst and McLachlan 1984). Preparations from the earliest fetal stages examined (F30–F32) were only viable for ~2 h at 35°C, after which time the ganglion and connective tissue began to deteriorate. At later stages (F38+), preparations were viable for ~8 h at 35°C (longest time attempted) and impalements were routinely held for >20 min.

INTRACELLULAR RECORDINGS OF SYMPATHETIC NEURONS. Neurons were impaled using high-resistance glass microelectrodes (80–200 MΩ) pulled on a Flaming-Brown puller (Sutter Instrument, Novarto, CA) and filled with 0.5 M KCl. Electrical properties were determined using bridge mode, discontinuous current clamp (DCC), or single electrode voltage clamp (SEVC) using either an Axoprobe-1A or an Axoclamp-2B amplifier (Axon Instruments, Union City, CA). Voltage or current records were digitized at 1–5 kHz using Spike2 (version 3.01) and Signal software (version 1.72; Cambridge Electronic Design, Cambridge, UK) on a PC running Windows NT, or Chart/Scope software (version 3.5, MacLab, ADI Instruments, Castle Hill, NSW, Australia) on a Power Macintosh computer (Apple Computers, Cupertino, CA). During DCC and SEVC, the headstage was continuously monitored and the cycling frequency adjusted to minimize the effects of electrode capacitance. The cycling frequency was 1.0–2.0 kHz for DCC and 2.0–3.5 kHz for SEVC. Digitized data were analyzed using Igor Pro (version 3.14, WaveMetrics, Lake Oswego, OR).

Resting membrane potential (RMP) was determined by measuring the difference between the potentials immediately before and after withdrawal of the microelectrode from the cell. Measurements of input resistance (\(R_{\infty}\)) and the major input time constant were made by injecting small hyperpolarizing current pulses (0.01–0.1 nA, 200–250 ms duration) through the recording electrode. Averages of 20 current steps were routinely used. The time constant of the cell (\(\tau, \text{ms}\)) was determined by fitting a single exponential to the onset of the voltage response to the current pulse between 20 and 80% of its final amplitude. Capacitance was derived from the time constant divided by the

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repolarization after the action potential until it returned to RMP). (mV) and half-width (ms) of the AP were measured together with the injections of depolarizing current from rest. The maximum amplitude made in bridge mode. Single APs were elicited by brief (10–20 ms) after hyperpolarization (AHP; Cassell and McLachlan 1987). For neurons were further discriminated as long afterhyperpolarizing (LAH) or not on the basis of the presence or absence of a prolonged duration after hyperpolarization (AHP; Cassell and McLachlan 1987). For measurement of action potential (AP) amplitudes, recordings were made in bridge mode. Single APs were elicited by brief (10–20 ms) injections of depolarizing current from rest. The maximum amplitude (mV) and half-width (ms) of the AP were measured together with the maximum amplitude (mV) and duration of the afterhyperpolarization (ms; measured from the point at which the cell passed RMP during repolarization after the action potential until it returned to RMP). For measurements of $I_{\text{SHP}}$ and $I_{\text{SLAP}}$ in SEVC, brief (10–20 ms) suprathereshold depolarization voltage steps resulted in a single “action current that corresponds to an unclamped action potential (Cassell et al. 1986; Jobling et al. 1993; Wang and McKinnon 1995). Peak amplitudes of $I_{\text{SHP}}$ and $I_{\text{SLAP}}$ were measured 10 ms after the action current. The time constant of the $I_{\text{SHP}}$ was determined by fitting a single exponential between 80 and 20% of the curve (Cassell and McLachlan 1987).

**Drug applications.** The action potentials of developing neurons either are initially dependent on Ca$^{2+}$ before becoming Na$^{+}$ dependent or they are Na$^{+}$ dependent from the onset of excitability (Spitzer 1991). The ionic dependence of early fetal neurons was examined using tetra-dotoxin (TTX, 1 μM; Alumane Labs, Jerusalem, Israel) to block Na$^{+}$-dependent channels and Cd$^{2+}$ (300 μM; ICN Biomedicals, Costa Mesa, CA) to block Ca$^{2+}$-dependent channels (Adams and Harper 1995; Davies et al. 1999). Solutions were changed by switching the perfusion line.

**Neuronal morphology.**

**Relative area of neuropil in topographically distinct regions of the celiac ganglion.** Celiac ganglia were removed from embryos (Carnegie stages 20–23), fetuses, neonates, and adults, fixed in Zamboni’s fixative (0.2% picric acid and 2% formaldehyde in 0.1 M phosphate buffer, pH 7.0), and processed for multiple-labeling immunohistochemistry (Anderson et al. 2001). Ganglia were dehydrated in ethanol (EtOH), cleared in DMSO, washed in 100% EtOH, and vacuum infiltrated at 46°C for ≥30 min in polyethylene glycol (PEG; MW 1000), before being embedded in PEG (MW 1450) in small cryomolds. Sections, 10–20 μm thick, were cut on a standard rotary microtome and placed into phosphate-buffered saline (PBS). Excess PBS was removed and the sections placed in 10% normal donkey serum (NDS) for 30 min. Sections were incubated in 10% NDS and primary antisera at room temperature for 48–72 h. Labeling for neurotensin (NT) was used to identify vasoconstrictor neurons, and labeling for somatostatin (Som) was used to identify neurons projecting to the enteric plexuses; labeling for tyrosine hydroxylase (TH) was used as an internal labeling control because nearly all celiac ganglion neurons contain TH regardless of their peptide content (Anderson et al. 2001; Costa and Furness 1984). Primary antibodies used were: sheep anti-NPY (Oliver/Blessing E2210/2; 1:1000) or rabbit anti-NPY (Incstar, Stillwater, MN, No. 550212; 1:1200), monoclonal mouse anti-Som (MRC Regulatory Peptide Group, Vancouver, Canada; code Soma S8; 1:1200) or rabbit anti-Som (Incstar; 1:100), and in some cases mouse anti-TH (Incstar, No. 105440; 1:1200) or rabbit anti-TH (Dr. J. Thibault, AS2–512, 1:200). After washing in PBS, secondary antibodies were applied for ≥2 h. Species-specific secondary antibodies (IgG) were raised in donkeys and conjugated with dicholortriazinyl amino fluorescein (DTAF), fluorescein isothiocyanate (FITC) or the indocarbocyanin dyes Cy3 or Cy5. All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, West Grove, PA. After further washing, sections were mounted on glass slides in carbonate-buffered glycerol (pH 8.6), and the coverslips were sealed using clear nail polish.

Sections were examined using conventional wide-field fluorescence or confocal microscopy. For conventional microscopy, images were collected using an Olympus AX70 microscope (Olympus, Tokyo, Japan) fitted with a Hamamatsu Orca cooled CCD camera (Hamamatsu Photonics, KK, Japan) and connected to a PowerMac G3 (Apple Computers) running IPLab Spectrum (version 3.2, Scansalytics, Fairfax, VA). Confocal microscopy was done using a BioRad MRC-1024 scanning laser confocal microscope (BioRad, Hemel Hempstead, UK) with a krypton/argon laser source fitted to an Olympus AX70 epifluorescence microscope and running under LaserSharp software (version 3.2, BioRad).

A stereological point-counting method (Howard and Reed 1998) was used to quantify changes in the proportional area of ganglion occupied by neuropil in lateral and medial regions of the developing celiac ganglion. Digital images of sections labeled for immunoreactivity to NPY, Som, and, in some cases, TH, were overlaid with a 25–35 point grid using Adobe Photoshop software (version 5.1, Adobe Systems, Mountain View, CA). Points intersecting with neurons, nonneuronal tissue, and neuropil were scored. Up to four samples from medial or lateral locations were averaged in each animal (see also Anderson et al. 2001).

**Neurobiotin-filled neurons.** During some intracellular impalements, Neurobiotin (0.5% wt/vol in 0.5 M KCl; Vector, Burlingame, CA) was included in the electrode filling solution so that neurons could be visualized after the completion of electrophysiological experiments. The location of neurons in the bilobed celiac ganglion was recorded as either in the medial two-thirds or in the lateral third of a lobe. At the completion of the electrophysiological recordings, ganglia were fixed in Zamboni’s fixative for 24–72 h and processed as whole mounts for multi-labeling immunohistochemistry as previously described (Gibbins et al. 1999; Jobling and Gibbins 1999). Briefly, picric acid was removed by washing in 80% EtOH before the tissue was further dehydrated in 100% EtOH and permeabilized in DMSO for 1–3 h. Tissue was then rehydrated through 80 and 50% EtOH before being washed in PBS (pH 7.0). Primary antisera for NPY and Som (as in the preceding text at twice the concentrations used for sections) were then applied for 48–72 h. After extensive washing in PBS, whole mounts were incubated in secondary antibodies overnight. Species-specific secondary antibodies (IgG), raised in donkeys and conjugated with DTAF, FITC, or Cy3 (see preceding text) were used to detect immunoreactivity to NPY and Som. Streptavidin conjugated to Cy5 (Jackson ImmunoResearch Laboratories) was used to detect Neurobiotin-filled cells. After 2–4 h washing in PBS, ganglia were mounted on glass slides in carbonate-buffered glycerol (pH 8.6).

Scanning laser confocal microscopy was used to analyze dye-filled neurons. Neurons first were assessed for their immunoreactivity to NPY or Som. To ensure antibodies had penetrated sufficiently through the whole ganglion, dye-filled neurons were only scored as lacking neuropotides if at least some adjacent neurons showed positive immunoreactivity. Dye-filled neurons were only included in the morphological analysis if individual dendrites were easily distinguishable, if they were not obscured by adjacent dye-filled neurons, and if the axon could be identified and followed to the edge of the ganglion. A low-magnification confocal through-focus series, which included all dendrites, was taken of each filled neuron with optical sections separated by 0.5–2.0 μm. A confocal through-focus series also was taken of the neuronal cell body at higher magnification using low gain settings to confirm that only one neuron had been filled and to provide a more precise measure of the cross-sectional and surface area of the soma. A single two-dimensional (2D) maximum-intensity projection image was generated from each confocal series using either Lasersharp or National Institutes of Health Image (NIH) software (version 1.61, Bethesda, MD). Measurements of cross-sectional area of the neuronal cell body (μm$^2$), number of primary dendrites (≥1 cell body diameter in length), and the total dendritic length (μm) were taken. Confocal through-focus series also were reconstructed on a PC using VoxBlast software for Windows (version 3.0, VayTek, Iowa City, IA). Threshold was optimized either for dendrites (low-
magnification series) or for the cell soma (high-magnification series), the surface area calculated and images of three-dimensional reconstructions rendered. The brightness and contrast of images was adjusted using Adobe Photoshop software.

TESTS OF MORPHOLOGICAL MEASUREMENTS AFTER DIFFERENT FIXATION AND MOUNTING TECHNIQUES. To test for any morphological changes that may accompany tissue processing of dye filled neurons, a conjugate of Dextran (10,000 MW), tetramethylrhodamine and biotin (“Mini-Ruby”; 20 μl in 2% 0.5 M KCl; Molecular Probes, Eugene, OR) was used to fill neurons in celiac ganglia from two adult guinea pigs. The ganglia were then mounted on glass slides in the same solution used during the experiments, and the coverslips were held in place using nail polish. A confocal through-focus series of each dye-filled neuron was captured before ganglia were removed from the slides and fixed in Zamboni’s fixative. Following fixation, ganglia were processed as for other experiments before they were re-mounted in PBS. Then a second confocal through-focus series was taken of each dye-filled neuron. Finally, ganglia were re-mounted in buffered glycerol and left overnight before a third confocal through-focus series of each dye-filled neuron was captured. A single 2D maximum-intensity projection image was generated from each confocal series, and NIH image software was used to measure the cross-sectional cell body area and total dendritic length. Three neurons sufficiently well filled for morphological analysis were followed throughout all the steps. None of these neurons underwent any significant shrinkage or any other morphological deformations with the fixation, processing, and mounting techniques used here.

STATISTICAL ANALYSIS. Development changes were analyzed with least-squares linear regression, with log-transformed weight used as a measure of developmental age. Means were compared with t-tests or multivariate ANOVA, while medians of strongly skewed data were compared with Mann-Whitney U tests. Frequency data were analyzed using χ² tests. All analyses were done with SPSS for Windows (version 9, SPSS, Chicago, IL). Data are presented as untransformed means ± SE, with n values referring to the number of neurons unless otherwise stated.

RESULTS
Development of electrical properties of celiac ganglion neurons

PASSIVE MEMBRANE PROPERTIES. Intracellular recordings were made from 177 neurons in 63 preparations of celiac ganglia from fetal, neonatal, and adult guinea pigs. The RMP of celiac

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FIG. 1. Relationships between increasing body weight of the animal and passive membrane properties. Weight (in g) is shown on a log scale. Approximate stages of development are indicated (F, fetal; Neo, neonate). The number of neurons (n), R² value, and significance of the regression (*P < 0.05) are indicated. Regression lines are shown with 95% confidence limits. A: the resting membrane potential (RMP, mV) of neurons became significantly more hyperpolarized during development (R² = 0.42, F(1,127) = 93.3, P < 0.0001). B: there was no significant change in input resistance (Rᵢᵢ, MΩ) during development (R² = 0.01, F(1,162) = 1.5, P = 0.2). C: the major cell input time constant (τₗ, ms) increased significantly during development (R² = 0.12, F(1,116) = 15.2, P < 0.0001). D: the apparent cell capacitance (derived from measured Rᵢᵢ and τₗ) significantly increased during development (R² = 0.16, F(1,116) = 22.7, P < 0.0001).
ganglion neurons became significantly more negative during development (Fig. 1A). At early fetal stages (F30–F35), the majority of neurons had RMPs around −35 mV while those from neonates had RMPs around −55 mV. In previous studies of developing sympathetic ganglia using intracellular recording techniques, neurons with RMPs outside published ranges for mature sympathetic neurons were not considered for analysis (e.g., Dryer and Chiappinelli 1985; Hirst and McLachlan 1984). However, in this study, half of the early fetal neurons with RMPs around −35 mV had input resistances $100 \, \text{M} \Omega$ (see following text; Fig. 1, A and B), suggesting that they were unlikely to have been damaged significantly during intracellular impalements. Therefore we have included immature neurons with RMPs less negative than −55 mV in the analyses that follow.

In contrast to RMP, the input resistance ($R_{in}$) of neurons did not change significantly during development (Fig. 1B). Overall, the mean input resistance was 119.0 ± 6.0 MΩ ($n = 169$). The major time constant ($\tau$) increased significantly during development, from ~7 ms at early fetal stages to ~11 ms at later stages (Fig. 1C). As a consequence, the apparent cell capacitance also showed a significant increase with age from ~60 pF at early fetal stages to ~100 pF at subsequent stages (Fig. 1D).

**ACTION POTENTIAL CHARACTERISTICS AND FIRING PROPERTIES.** The peak amplitude of the AP, the potential at which this peak was reached, and the AP half-width were measured in neurons that generated an AP in response to a 10- to 20-ms depolarizing step. Although the peak amplitude of the AP increased significantly during development (Fig. 2A), the potential at which this was reached (between 10 and 30 mV) did not change significantly ($R^2 = 0.03$, $F_{(1,61)} = 1.61$, $P = 0.2$). Thus the increase in peak AP amplitude is likely to reflect the fact that...

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**Fig. 2.** Relationships between increasing body weight of the animal and characteristics of the action potentials (APs) and afterhyperpolarizations (AHP). Weight (in g) is shown on a log scale. Approximate stages of development are indicated (F, fetal; Neo, neonate). The number of neurons ($n$), $R^2$ value, and significance of the regression (*$P < 0.05$) are indicated. Regression lines are shown with 95% confidence limits. A: the peak amplitude of the AP (mV) increased significantly during development ($R^2 = 0.46$, $F_{(1,86)} = 72.2$, $P < 0.001$). B: there was a significant decrease in the duration of the AP (AP half-width, ms) during development ($R^2 = 0.14$, $F_{(1,38)} = 5.9$, $P = 0.02$). C: during development, the amplitude of the AHP increased significantly ($R^2 = 0.42$, $F_{(1,74)} = 52.6$, $P < 0.0001$). When analyzed separately, both tonic ($R^2 = 0.20$, $F_{(1,23)} = 5.8$, $P = 0.025$) and long afterhyperpolarizing (LAH) neurons ($R^2 = 0.58$, $F_{(1,31)} = 42.1$, $P < 0.0001$) showed significant increases in AHP amplitude during development. The regression line is shown for the total neuronal population. D: the duration of the AHP (ms) significantly increased during development in tonic neurons ($R^2 = 0.22$, $F_{(1,22)} = 6.3$, $P = 0.02$), but significantly decreased in LAH neurons ($R^2 = 0.23$, $F_{(1,28)} = 8.6$, $P = 0.007$).
the RMP becomes more negative with age (Fig. 1A). Finally, there was a small but significant decrease in the AP half-width during development as reported in other developing neurons (Fig. 2B) (Spitzer and Ribera 1998).

In adult sympathetic neurons, differences in neuronal firing properties result from the differential expression of voltage-dependent K+ channels and Ca2+-dependent K+ channels (Adams and Harper 1995). Therefore the identification of neurons with different firing properties provided the first step in determining when the mature combinations of channels are expressed during fetal development. We therefore investigated the responses of developing neurons to maintained depolarizing current steps. The majority of early fetal neurons (34 of 50) generated only a single AP with a short amplitude and long duration in response to a depolarizing voltage step (Figs. 3 and 4A). This presumably reflects the smaller electrical driving force driving Na+ and the inactivation of some Na+ channels at RMP in the range −30 to −40 mV. Increasing the magnitude and duration of the current step further did not elicit any additional APs. This finding also suggests that channels underlying differences in firing properties are not expressed at these early fetal stages of development. Six early fetal neurons generated multiple APs from rest, while 10 others revealed a single shunted AP only if the neuron was hyperpolarized. Since the majority of early fetal neurons only elicited a single AP in response to depolarization, the ionic dependence of seven immature neurons was examined using 1 μM TTX (voltage-dependent Na+ channel blocker) and/or 300 μM Cd2+ (non-specific Ca2+ channel blocker). The APs of two neurons were completely blocked with Cd2+ alone (Fig. 3A), another two were blocked with TTX alone (Fig. 3B), while three required the combined presence of TTX and Cd2+ (Fig. 3C). These results suggest that the APs of some neurons are initially Na+ dependent, while others are initially dependent on Ca2+.

From mid fetal stages onward, two main types of neurons could be identified by their firing properties in response to depolarizing current injections, as has been previously reported in adult guinea pig sympathetic neurons (Cassell and McLachlan 1987; Cassell et al. 1986). Approximately one-third of mid fetal neurons (12 of 32; Fig. 5A) and one-third of late fetal, neonatal and adult neurons (22 of 74; Fig. 7A) fired APs throughout the duration of a depolarizing current injection, similar to adult tonic (slowly adapting) neurons (Cassell et al. 1986; McLachlan and Meckler 1989). At all developmental stages, these tonic-firing neurons were primarily located within medial regions of the celiac ganglion, as seen in mature guinea pigs (McLachlan and Meckler 1989).

Another third of the mid fetal neurons (11 of 32; Fig. 6A) and more than half of the neurons at late fetal, neonatal, and adult stages (42 of 74; Fig. 8A) adapted rapidly at the onset of a suprathreshold depolarizing current step, as occurs in adult phasic neurons (Cassell et al. 1986). At mid fetal and later stages of development, 64 and 76% of the phasic neurons, respectively, had a LAH lasting ≥1 s, typical of adult LAH neurons (Cassell et al. 1986; McLachlan and Meckler 1989). Regardless of developmental stage, the majority of phasic-firing neurons were located in the lateral regions of the celiac ganglion as seen in mature guinea pigs (McLachlan and Meckler 1989).

The remaining 9 of the 32 mid fetal neurons examined resembled early fetal neurons as they had single, small amplitude APs and could not be definitively classified by their firing properties. In contrast, only 8 of the 74 neurons from late fetal and subsequent stages could not be classified by their firing properties alone (see also, Cassell et al. 1986; Keast et al. 1993; McLachlan and Meckler 1989; Stebbing and Bornstein 1993).

DEVELOPMENT OF THE AHP. In mature guinea pigs, most sympathetic neurons have a prominent AHP that is largely due to the presence of a Ca2+-dependent K+ current, IAHP (sometimes called gKCa1) (Cassell and McLachlan 1987; Cassell et al. 1986). In addition to this current, LAH neurons also have a second Ca2+-dependent K+ current that is responsible for the prolonged phase of the AHP (IAP or gKCa2) (Cassell and McLachlan 1987; Jobling et al. 1993). Here we consider the developmental appearance of each phase of the AHP. A summary of the results obtained using current-clamp recordings, which show the combined effects of these two currents, is shown for neurons at different stages of development in Table 2.
Fast AHP ($I_{AHP}$). The amplitude and duration of the AHP was determined in neurons where an AP was generated in response to a brief (10–20 ms) suprathreshold current step. In 19 of 21 early fetal neurons, where no AP could be generated during a depolarizing step, an AHP was observed after the AP generated at the end of a hyperpolarizing current step (i.e., off the “anode break”). Overall, there was a significant increase in AHP amplitude from ~7 mV at mid fetal stages to ~15 mV at subsequent stages (Fig. 2C; Table 2). There was a significant increase in the duration of the AHP in tonic neurons from ~245 ms at mid fetal stages to ~310 ms at all later stages (Fig. 2C; Table 2).

The amplitude and time constant of the current underlying this AHP, $I_{AHP}$, were measured in early fetal and mid fetal neurons. In early fetal neurons, $I_{AHP}$ ranged from 17 to 45 pA (mean 29.7 ± 8.2 pA, $n = 3$) while the time constant ranged from 13 to 168 ms (91.5 ± 44.8 ms, $n = 3$; Fig. 4Be). In mid fetal tonic neurons, the peak amplitude of $I_{AHP}$ ranged from 49 to 230 pA (108.0 ± 23.4 pA, $n = 7$) while the time constant ranged from 28 to 148 ms (95.2 ± 24.7 ms, $n = 7$; Fig. 5Ac).

Slow AHP ($I_{sAHP}$). No evidence of slow AHPs lasting ≥1 s was found in early fetal neurons. From mid fetal stages, neurons with slow AHPs characteristic of adult LAH neurons were present. The duration of the AHP in LAH neurons decreased by ~50% during development from ~3.5 s to ~2.5 s (Fig. 2D; Table 2). The peak amplitude of $I_{sAHP}$ in mid fetal neurons ranged from 20 to 60 pA (41.3 ± 8.4 pA, $n = 4$), which was somewhat less than that reported for LAH neurons from mature guinea pigs (100 pA, Cassell and McLachlan 1987; 56 pA, Martínez-Pinna et al. 2000). However, the decay time constant of the underlying current in mid fetal LAH neurons (1.6 ± 0.1 s, $n = 4$; Fig. 6A) was similar to that reported for mature guinea pigs (1.4 s, Cassell and McLachlan 1987; 1.2 s, Martínez-Pinna et al. 2000). This suggests that the reduction in the duration of the AHP during development is unlikely to be due to changes in the kinetics of $I_{sAHP}$.

**CURRENT-VOLTAGE RELATIONSHIPS.** When early and mid fetal neurons were injected with depolarizing or hyperpolarizing currents small enough to alter the membrane potential by 10–20 mV, the membrane potential often shifted back toward rest during the current injection (Fig. 4Ab). This sag in the membrane potential suggests the deactivation of a voltage-dependent current that is active around RMP. Such a voltage-dependent current is characteristic of the time-dependent rectification produced by the closure of M channels (Adams and Harper 1995; Brown 1988; Brown and Adams 1980; Cassell et al. 1986). When neurons were held positive to ~60 mV in voltage clamp, hyperpolarizing voltage steps showed inward and outward relaxations typical of M current (Figs. 4Be and 5Ac). This current was deactivated below ~60 mV as found in other sympathetic ganglia (Brown et al. 1982; Cassell et al. 1986; Jobling and Gibbins 1999; Wang and McKinnon 1995). The peak amplitude of $I_{M}$, measured when stepped from...
240 to 260 mV, was 30 pA in three early fetal neurons. Although all mid fetal tonic neurons were observed to have a sag in the voltage trace, the amplitude of $I_M$ was only small compared with adults (Cassell et al. 1986; Coggan et al. 1994). The amplitude of $I_M$ in four mid fetal tonic neurons was 30 pA (mean 32.8 ± 9.2 pA, $n = 5$). Two mid fetal LAH neurons had $I_M$ amplitudes of 16 and 74 pA.

Half of the early fetal neurons, but only 26% of mid fetal neurons, showed a noticeable depolarizing sag in their voltage trace during large current injections that hyperpolarized the neuron below 2100 mV (Figs. 4Bf and 5Af; Table 3). In voltage clamp, slowly activating inward currents were observed when the holding potential was stepped below 2100 mV (Figs. 4Bg and 5Ag). These currents resembled $I_H$ (sometimes called $I_Q$ or $I_f$), which has previously been described in...

Plots of current amplitude against steady-state voltage responses revealed inward (or anomalous) rectification when neurons were hyperpolarized below $-90$ mV in about one-third of early fetal neurons (Fig. 4Bb), $>90\%$ of mid fetal tonic neurons (Fig. 5Ab, see also Fig. 7Ab), but $<30\%$ of mid fetal

![FIG. 6. Electrical and morphological properties of a mid fetal (F44) LAH neuron. Aa: current-clamp records showing changes in membrane potential (top) in response to depolarizing and hyperpolarizing current steps (bottom). This neuron only discharged APs at the onset of suprathreshold depolarizing steps. Ab: current-voltage relationship. Ac: current-clamp record of AP and the LAH (top), evoked after a brief suprathreshold depolarizing current step (bottom). Ad: voltage-clamp record of an unclamped action current and tail currents underlying the AHP (top, filtered at 450 Hz), generated after a brief (10 ms) depolarizing voltage step (bottom). Ba: rendered 3D reconstruction of low-magnification confocal throughput-series of the same neuron whose electrical properties are shown in A. Threshold was optimized for dendrites. *, axon. Scale bar = 20 \mu m. Bb: rendered 3D reconstruction of high-magnification confocal throughput-series of the neuronal soma.]

TABLE 2. Current-clamp measurements of amplitude and duration of afterhyperpolarization (AHP) at different stages of development

<table>
<thead>
<tr>
<th></th>
<th>Mid Fetal (F40–F45)</th>
<th>Late Fetal (F46+)</th>
<th>Neonatal (P0–P13)</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHP amplitude, mV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All neurons</td>
<td>7.5 ± 0.9 (25)</td>
<td>16.1 ± 3.1 (7)</td>
<td>15.0 ± 0.9 (35)</td>
<td>15.4 ± 1.1 (12)</td>
</tr>
<tr>
<td>Tonic neurons</td>
<td>10.5 ± 1.5 (11)</td>
<td>16.4 ± 3.4 (4)</td>
<td>15.2 ± 2.5 (9)</td>
<td>15.6 ± 1.4 (3)</td>
</tr>
<tr>
<td>LAH neurons</td>
<td>5.9 ± 0.9 (6)</td>
<td>4.0 (1)</td>
<td>15.1 ± 1.1 (18)</td>
<td>16.3 ± 1.7 (7)</td>
</tr>
<tr>
<td>Phasic neurons</td>
<td>6.8 ± 2.0 (3)</td>
<td>26.4 (1)</td>
<td>14.5 ± 1.6 (7)</td>
<td>14.8 (1)</td>
</tr>
<tr>
<td>AHP duration, ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All neurons</td>
<td>1201.6 ± 337.2 (22)</td>
<td>281.5 ± 42.7 (6)</td>
<td>1495.5 ± 207.4 (34)</td>
<td>1807.7 ± 407.1 (12)</td>
</tr>
<tr>
<td>Tonic neurons</td>
<td>244.6 ± 35.0 (11)</td>
<td>313.0 ± 28.4 (4)</td>
<td>488.1 ± 65.0 (9)</td>
<td>348.0 ± 75.8 (3)</td>
</tr>
<tr>
<td>LAH neurons</td>
<td>3695.0 ± 187.0 (6)</td>
<td>—</td>
<td>2471.9 ± 192.0 (18)</td>
<td>2885.7 ± 230.2 (7)</td>
</tr>
<tr>
<td>Phasic neurons</td>
<td>295.0 ± 50.6 (3)</td>
<td>90 (1)</td>
<td>279.9 ± 33.9 (7)</td>
<td>410 (1)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Parentheses enclose number of neurons. LAH, long AHP.
LAH neurons (Fig. 6Ab, see also Fig. 8Ab; Table 3). The voltage-dependent K\(^+\) current, \(I_{Kp}\), responsible for this rectification has been identified in many autonomic neurons where it is predominantly restricted to tonic-firing neurons (Adams and Harper 1995; Cassell and McLachlan 1987; Cassell et al. 1986; Keast et al. 1993; Wang and McKinnon 1995).

After neurons were hyperpolarized to potentials below \(-60\) mV, a prolonged delay was often seen in the voltage trace as the membrane returned to rest (Fig. 5Aa). This delay or “notch” has been described in mature sympathetic neurons, where it is due to the activation of a transient outward A current (\(I_A\)) (Adams and Galvan 1986; Cassell et al. 1986; Connor and Stevens 1971; Galvan and Sedlmeir 1984; Wang and McKinnon 1995). Around half of the early fetal neurons and 80% of mid fetal tonic neurons appeared to express \(I_A\) (Table 3). In 10 mid fetal tonic neurons that were voltage clamped at rest, a transient outward \(I_A\) was seen when the holding potential returned to rest (around \(-50\) mV) after being held below \(-60\) mV. The time constant of \(I_A\) inactivation in these neurons ranged from 7 to 20 ms (12.9 ± 2.1 ms, \(n = 6\)), which is less than that reported for mature guinea-pig sympathetic tonic neurons (mean 22.1 ms, \(n = 17\)) (Cassell et al. 1986).

An outward current, slower and more prolonged than \(I_A\) (slow \(I_A\)) was observed in 41% of early fetal neurons, including some with single shunted action potentials (Fig. 4B, f and g), and 40% of mid fetal tonic neurons (Fig. 5A, f and g; Table 3). This current was not seen in LAH or phasic neurons at mid fetal stages, reflecting the situation in mature guinea-pigs (Table 3) (Cassell et al. 1986). Similar currents have been observed in developing rat sympathetic neurons (\(I_{D2}\)) (McFarlane and Cooper 1992) where, in mature animals, its expression also is restricted to tonic neurons (\(I_{D2}\)) (Wang and McKinnon 1995).

### Development of neuronal morphology

#### PROPORTION OF AREA OCCUPIED BY NEUROPILOh We used the relative area of the celiac ganglion occupied by neuropil as an indicator of dendritic growth in our stereological analysis of the medial and lateral regions at different stages of development (Fig. 9A). At late embryonic stages, very little neuropil was observed in either medial (area of neuropil: 4 ± 4% of total sample area, \(n = 3\) embryos) or lateral regions (6 ± 4%; \(n = 3\) embryos). The relative area occupied by neuropil significantly increased throughout development in both medial and lateral regions. However, the rate of increase observed was greater in medial regions (\(P < 0.05\)) so that by adult stages, the relative area occupied by neuropil in medial regions (50 ± 2%; \(n = 5\) animals) was significantly higher than that in lateral regions (33.6 ± 4.1%; \(n = 5\) animals, \(P < 0.05\); Fig. 9A).

#### TABLE 3. Proportion of neurons with proposed ionic currents

<table>
<thead>
<tr>
<th></th>
<th>Immature</th>
<th>Immature†</th>
<th>Tonic</th>
<th>LAH</th>
<th>Phasic</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I_M)</td>
<td>15/21 (71)</td>
<td>7/11 (64)</td>
<td>15/15 (100)</td>
<td>5/7 (71)</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>(I_{H})</td>
<td>9/17 (53)</td>
<td>1/5 (20)</td>
<td>3/10 (30)</td>
<td>1/6 (17)</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td>(I_A)</td>
<td>10/19 (53)</td>
<td>4/8 (50)</td>
<td>12/15 (80)</td>
<td>4/7 (57)</td>
<td>2/3 (67)</td>
</tr>
<tr>
<td>Slow (I_A)</td>
<td>9/22 (41)</td>
<td>0/7 (0)</td>
<td>6/15 (40)</td>
<td>0/7 (0)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>(I_{Kp})</td>
<td>5/17 (29)</td>
<td>0/5 (0)</td>
<td>11/12 (92)</td>
<td>2/7 (29)</td>
<td>0/3 (0)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages. * Gestational age range for animals used in this part of the study. † Could not be classified according to firing properties.

#### FIG. 7. Electrical and morphological properties of a late fetal (F49) tonic neuron.

**Aa:** current-clamp record showing continuous discharge of APs (top) in response to a suprathreshold depolarizing current step (bottom). **Ab:** current-voltage relationship showing marked inward rectification below \(-90\) mV. **Ac:** 10-ms depolarizing current step elicited a single AP that was followed by an AHP with a duration <500 ms. Trace filtered at 150 Hz. **B:** rendered 3D reconstruction of a low-magnification confocal through-series of the neuron. Threshold was optimized for dendrites. * axon. Scale bar = 50 \(\mu m\).
Neurons, some of whose electrical properties had been analyzed (Figs. 5B, 6B, 7B, and 8B), were examined. It was difficult to achieve reliable fills of early fetal neurons, presumably due to the short impalement times. Therefore the analysis of dendritic fields is largely restricted to mid fetal and subsequent stages. There was a small but significant increase in the number of primary dendrites (Fig. 9B) and total dendritic length (Fig. 9C) during these stages. Consistent with the stereological analyses, by late fetal stages, medially located neurons had more primary dendrites (12.4 ± 1.4, n = 7) and greater total dendritic lengths (1,681 ± 330.0 μm, n = 7) compared with laterally located neurons with 6.6 ± 1.7 (n = 5) primary dendrites and total dendritic lengths of 674.4 ± 246.1 μm (n = 5).

There was a dramatic increase in the cross-sectional area of neuronal cell bodies during development from 170 μm² at early fetal stages to >1,000 μm² at neonatal stages (Fig. 9D). The cell body cross-sectional area of neurons was similar regardless of their topographical location within the ganglion (Mann-Whitney U test = 2,386.5, P = 0.7, n = 147). When the morphological and electrical properties of individual neurons were determined, the surface area and capacitance were calculated. Overall there was no significant correlation between the total surface area (neuronal soma and dendritic field) of a neuron and the derived input capacitance (Fig. 9E). However, the increasing surface area of neuronal soma was strongly correlated with an increase in the derived input capacitance (Fig. 9F).

Electrophysiological class, morphology, and neuropeptide content. Using a subset of neurons described above, differences between the morphology of dye-filled tonic and LAH neurons were examined from mid fetal through to neonatal stages. As previously described in mature guinea pigs (Gibbins et al. 1999; Keast et al. 1993), tonic neurons were located in medial regions while LAH neurons were located in lateral regions (χ² = 5.3, df = 1, P = 0.02, n = 53 neurons). While no differences were found in the cross-sectional areas of neuronal cell bodies (tonic, 844.5 ± 103.2 μm², n = 16; LAH, 1,159.2 ± 100.0 μm², n = 18; F₁,16 = 0.91, P = 0.4), tonic neurons had more primary dendrites (12.9 ± 1.0, n = 12) compared with LAH neurons (7.3 ± 0.6, n = 16; F₁,23 = 19.7, P < 0.001) as well as greater total dendritic lengths (tonic, 2,299.3 ± 345.6 μm, n = 12; cf. LAH, 848.2 ± 65.6 μm, n = 16; F₁,25 = 23.9, P < 0.001; Fig. 9D). Consequently, the soma of tonic neurons formed a significantly smaller proportion of the total neuronal surface area (6.5 ± 0.9%, n = 9) compared with LAH neurons (16.6 ± 2.2%, n = 12; t-test, df = 19, P = 0.001).

The neuropeptide content of 101 dye-filled neurons at fetal and neonatal stages was determined. At mid fetal stages, 72% of neurons without NPY-IR, with or without Som-IR, were located in medial regions while the remaining neurons were located in lateral regions (n = 32). Across all stages examined, only 1 of 17 tonic neurons expressed NPY-IR, while 6 of 10 LAH neurons contained NPY-IR. Combined analysis of neurons from fetal and neonatal stages revealed that the cell body cross-sectional area of neurons without NPY-IR, many of which contained Som-IR, was only marginally greater than neurons with NPY-IR (Mann-Whitney U = 371.5, P = 0.05, n = 94). The number of primary dendrites on neurons with and without NPY-IR was not significantly different (Mann-Whit-
ney $U = 78.0, P = 0.08, n = 53$). In contrast, neurons without NPY-IR, including those with Som-IR, had significantly greater total dendritic lengths than those with NPY-IR ($U = 24.0, P = 0.003, n = 42$; Fig. 9C).

Overall the correlations between electrophysiological, morphological, and neurochemical properties of fetal neurons reflect those previously published for celiac ganglion neurons from mature guinea pigs. Thus NPY-IR neurons corresponded to LAH neurons with small dendritic fields, while Som-IR neurons corresponded to tonic neurons with large dendritic fields. Nevertheless neonatal neurons of all classes were still only about two-thirds the size of neurons.
in adult celiac ganglia (Boyd et al. 1996; Gibbins et al. 1999; Keast et al. 1993).

**DISCUSSION**

We have shown that different functional subpopulations of celiac ganglion neurons can be distinguished by their electrical and morphological properties from mid fetal stages of development. These distinctions occur after the neurochemical phenotypes of the neurons and the topographical organization of the celiac ganglion have been established (Anderson et al. 2001) but long before the neurons finish growing. The differentiation of these neurons involves the sequential expression of various K⁺ channels accompanied by divergent growth patterns of their dendritic trees. Furthermore each major functional class of neuron seems to develop directly from a topographically distinct subset of precursors.

**EARLY FETAL NEURONS EXPRESS DIFFERENT COMBINATIONS OF K⁺ CHANNELS.** Adult celiac ganglion neurons have characteristic patterns of expression of K⁺ channels. Most notably, M current is largely restricted to phasic/LAH neurons while A current regulates AP discharge in tonic neurons but not LAH neurons (Cassell and McLachlan 1987; Cassell et al. 1986; Wang and McKinnon 1995). In contrast with adult neurons, small M currents were detected in most early fetal neurons and in both phasic and tonic firing neurons at mid fetal stages. Consequently there must be a significant increase in M-current expression in phasic/LAH neurons but not tonic neurons during the later stages of fetal development. In mature sympathetic neurons, M-current is thought to exert a major influence on firing properties by reducing the rate of action potential generation (Adams and Harper 1995; Wang and McKinnon 1995). The low level of expression of M-current in developing celiac ganglion neurons suggests that it has only a limited influence on their firing properties. At early fetal stages, 40% of celiac ganglion neurons expressed the slow A-current. By mid fetal stages, the slow A-current was restricted to tonic neurons, suggesting that the early expression of this current provides the first indication that a neuron is destined to develop the tonic-firing phenotype.

In contrast to the early expression of M and A currents, the \( I_{\text{AHP}} \) responsible for the LAH was not detected until mid fetal stages. This explains the relatively late stage at which LAH neurons could be identified by functional criteria. The late expression of \( \text{Ca}^{2+} \)-dependent K⁺ channels also has been reported in other systems (Ahmed et al. 1986; Dryer 1994, 1998; Martin-Caraballo and Greer 2000). It has been suggested previously that ion channels required for basic neuronal excitability (such as voltage-dependent \( \text{Na}^+ \), \( \text{Ca}^{2+} \), and K⁺ channels) are established relatively early during development and are not influenced by external factors (Dryer 1994; Ribera and Spitzer 1992). However, the developmental expression of ion channels involved in the fine control of neuronal firing behavior (such as the \( \text{Ca}^{2+} \)-dependent K⁺ channel \( I_{\text{AHP}} \), as well as \( I_A \) and \( I_m \)) are likely to be influenced by extrinsic factors including the local environment, synaptic inputs, and targets (Barish 1995; Dryer 1994, 1998; McFarlane and Cooper 1992; Raucher and Dryer 1994, 1995). If so, the sequential expression of ion channels during development of celiac ganglion neurons implies the presence of multiple factors acting in a time-dependent way to regulate their differentiation.

**ELECTROPHYSIOLOGICAL AND MORPHOLOGICAL PHENOTYPES ARE ESTABLISHED DURING THE SAME DEVELOPMENTAL PERIOD.** The celiac ganglion neurons developed their characteristic electrical and morphological phenotypes in parallel, mainly during the mid fetal period. Such parallel development has been reported widely in other neurons (Allan and Greer 1997a,b; Dekkers et al. 1994; Kandler and Friauf 1995; Martin-Caraballo and Greer 1999; Phelan et al. 1997; Vincent and Tell 1999; Warren and Jones 1997). However, the electrical properties of celiac ganglion neurons did not change after they were established at mid fetal stages, whereas neurons continued to increase in size. Therefore as the neurons grow during late fetal and neonatal development, there must be continued regulated synthesis of phenotypically appropriate channels to match the on-going production of new cell membrane.

Much of the growth of the neurons involves the dendritic tree as well as the soma. Nevertheless developmental increases in apparent cell capacitance were correlated much more strongly with somatic surface area rather than with the total surface area of the neurons including their dendrites. The simplest interpretation of this observation is that most of the electrical properties we recorded arose from the somatic membrane with relatively little contribution from the dendrites. This is surprising since it is generally thought that mature autonomic neurons, which are significantly larger than the fetal neurons, are electrotonically compact (Adams and Harper 1995).

While celiac ganglion neurons from medial and lateral regions showed similar developmental increases in somatic size, the dendritic trees of neurons located in medial regions of the ganglion increased their total length at a greater rate than those in lateral regions. This differential growth results in tonic neurons in the medial regions of the ganglion bearing larger dendritic trees than LAH neurons in the lateral regions of the ganglion. The dissociation of somatic and dendritic growth...
rates has been reported previously in motor neurons of postnatal rats (Nunez-Abades and Cameron 1995) and suggests that somatic size and dendritic size may be regulated independently during development.

The medially located tonic-firing neurons in the mature guinea pig celiac ganglion are unusual in that they receive convergent synaptic inputs from neurons projecting from the gut wall (enteric intestinofugal neurons) in addition to synaptic inputs from preganglionic neurons projecting from the spinal cord (Kreulen and Szurszewski 1979; McLachlan and Meckler 1989; Meckler and McLachlan 1988). Previous studies on developing autonomic neurons have demonstrated a close temporal relationship between dendritic outgrowth and synapse formation (Dryer 1994; Hirst and McLachlan 1986; Rubin 1985a–c). Therefore we predict that synaptic inputs are established during the same developmental period in which differential growth of the dendrites is occurring, i.e., from early to mid fetal stages of development, and that the increased dendritic growth rate of the medial neurons is related to the arrival of additional peripheral inputs, which smaller lateral neurons lack. If this prediction is borne out, the initiation of the differential expression of at least some K+ channels, such as those responsible for the A current, may well precede synaptogenesis in the celiac ganglion.

In conclusion, we have shown that the differentiation of electrical and morphological properties of sympathetic neurons in the celiac ganglion follows the development of their neurochemical phenotype. The selective expression of K+ channels follows a step-wise sequence that occurs simultaneously with the differential growth of the dendritic trees of specific populations of neurons innervating the vasculature or the enteric plexuses. This sequential development combined with the dissociation between somatic and dendritic growth of the neurons strongly implies that many of these phenotypic traits can be independently regulated. Thus our data provide strong circumstantial evidence that the development of the full phenotype of different functional classes of autonomic final motor neurons is a multi-step process, likely to involve a regulated sequence of trophic interactions.

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DEVELOPMENT OF SUBPOPULATIONS OF AUTONOMIC NEURONS


