Changes in Somatodendritic Morphometry of Rat Oculomotor Nucleus Motoneurons During Postnatal Development

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ABSTRACT
This work investigates the somatodendritic shaping of rat oculomotor nucleus motoneurons (Mns) during postnatal development. The Mns were functionally identified in slice preparation, intracellularly injected with neurobiotin, and three-dimensionally reconstructed. Most of the Mns (~85%) were multipolar and the rest (~15%) bipolar. Forty multipolar Mns were studied and grouped as follows: 1–5, 6–10, 11–15, and 21–30 postnatal days. Two phases were distinguished during postnatal development (P1–P10 and P11–P30). During the first phase, there was a progressive increase in the dendritic complexity; e.g., the number of terminals per neuron increased from 26.3 (P1–P5) to 47.7 (P6–P10) and membrane somatodendritic area from 11,289.9 μm² (P1–P5) to 19,235.8 μm² (P6–P10). In addition, a few cases of tracer coupling were observed. During the second phase, dendritic elongation took place; e.g., the maximum dendritic length increased from 486.7 μm (P6–P10) to 729.5 μm in adult Mns, with a simplification of dendritic complexity to values near those for the newborn, and a slow, progressive increase in membrane area from 19,235.8 μm² (P6–P10) to 24,700.3 μm² (P21–P30), while the somatic area remained constant. In conclusion, the electrophysiological changes reported in these Mns with maturation (Carrascal et al. [2006] Neuroscience 140:1223–1237) cannot be fully explained by morphometric variations; the dendritic elongation and increase in dendritic area are features shared with other pools of Mns, whereas changes in dendritic complexity depend on each population; the first phase paralleled the establishment of vestibular circuitry and the second paralleled eyelid opening. J. Comp. Neurol. 514:189–202, 2009. © 2009 Wiley-Liss, Inc.

Indexing terms: postnatal development; oculomotor system; motoneurons; 3D reconstruction; slice preparation; rat

Neuronal dendritic trees are shaped during postnatal development. Local environmental signals trigger the processes of arborization and elongation of the growth cones located in the terminal segments of the dendritic trees (van Pelt and Uylings, 2002; Jan and Jan, 2003). Studies of these postnatal anatomical changes in motoneurons (Mns) are restricted to the hypoglossal nucleus and spinal cord. Studies on spinal Mns concluded that there are no changes in the number of dendritic branches with postnatal development, and the increase in dendritic surface area is accomplished by the elongation (preferentially of the terminal branches rather than preterminal ones) and the increase in thickness of existing branches (Ulfhake and Cullheim, 1988; Ulfhake et al., 1988; Ramirez and Ulfhake, 1991; Li et al., 2005). In contrast, phrenic Mns show a transient decrease in dendritic complexity and an increase in the length of branches for all order of the dendritic tree during postnatal maturation (Cameron et al., 1991; Greer and Funk, 2005). Furthermore, a pioneering study quantified changes in the dendritic tree with the temporal course of postnatal development in genioglossal Mns and found that the process was not continuous (Nunez-Abades et al., 1994; Nunez-Abades and Cameron, 1995). These Mns exhibit the most complex dendritic tree at birth; two phases were distinguished. During the first phase (up to 13–15 postnatal days), the dendritic diameter and surface area do not increase, but a redistribution of the preexistent membrane allows dendritic elongation. During the second phase (beyond 13–15 days old), the dendritic surface area increases twofold by both an enlargement in dendritic diameter of all branch orders and the generation of new terminal branches. These latter studies indicated that a systematic characterization of dendritic tree maturation in different Mn populations is required to under-
stand better the rules guiding this process and to recognize how dendritic shape could be linked to the maturation of the neural circuit (signals from the synaptic inputs and/or target arriving at the neuron in construction) and could be correlated with the electrophysiological properties.

Another anatomical characteristic that could be modified postnatally is somal size. Increases in the somal area have been reported in spinal (Ulfhake and Gulheim, 1988; Cameron et al., 1991) and brainstem (Honma et al., 2002; Smith et al., 2005) Mns. In contrast, geniculossomal Mn somal dimensions are achieved by birth (Nunez-Abades et al., 1994; Nunez-Abades and Cameron, 1995). It emerges from these latter studies that different signals could guide the growth of dendrites and soma.

On the other hand, studies of phrenic and geniculossomal Mns have reported the surprising fact that increases in cell surface do not underlie modifications in passive membrane properties but may result from a reduction in specific membrane resistance (Cameron and Nunez-Abades, 2000). For these populations, it has been suggested that proliferation and redistribution of synaptic inputs (Nunez-Abades et al., 2000) and/or potassium channels (Cameron and Nunez-Abades, 2000) might support the decrease in input resistance with age. In recent studies (Carrascals et al., 2005, 2006), we have reported changes in passive and active membrane electrophysiological properties in rat oculomotor nucleus Mns during postnatal development. Input resistance and time constant diminish drastically within a short time after birth (1–5 days postnatally). Could these changes be attributable to an increase of cell surface or be dependent on other factors, e.g., an increase in the density of leak potassium channels?

In the early developmental stages, Mns are coupled by gap junctions. About 25% of rat phrenic and lumbar spinal cord Mns are coupled at embryonic and neonatal periods (Walton and Navarrete, 1991; Martin-Caraballo and Greer, 1999). This arrangement facilitates the sharing of synaptic currents and/or intercellular messengers that may be involved in controlling neurogenesis, differentiation, and/or circuit formation (Montoro and Yuste, 2004). Electrotoc coupling between adjacent developing rat lumbar Mns is restricted to those innervating the same muscle and does not occur between Mns innervating antagonistic muscles (Walton and Navarrete, 1991). The rate of electrotoc coupling decreases postnatally in both lumbar (Walton and Navarrete, 1991) and brainstem (Mazza et al., 1992) Mns in the rat. Whether the coupling between Mns is also present in other pools, such as those of the oculomotor nucleus, is not known.

In homeotherms, the somatodendritic morphology of oculonuclear Mns is constant across species (Evinger, 1988). In particular, abducens and oculomotor nucleus Mns of adult rats have been intracellularly labeled and reconstructed (Durand, 1989a,b). The somata (23–40 μm in diameter) give rise to two to five primary dendrites, which branch into fine dendrites extending between 500 and 800 μm from the soma. The organization and extension of the dendritic trees depend on the location of the somata within the nucleus. Because studies on postnatal anatomical changes in oculonuclear Mns have not yet been performed, the present work investigated the following questions in the rat oculomotor nucleus Mns: Is the growth of the dendritic trees a continuous process, or is it characterized by periods? Could modifications in somal surface area be dissociated from those in the dendritic tree? Could the electrophysiological changes be correlated with dendritic and somal shaping? Are oculomotor nucleus Mns coupled at birth? Finally, could the maturation process of these Mns correspond with that of the oculomotor system? To investigate these questions, we quantified, in identified oculomotor nucleus Mns, the changes in somatodendritic morphology and percentage of coupled cells during postnatal development.

**MATERIALS AND METHODS**

Experiments were carried out on Wistar rats (5–150 g) of either sex from 1 to 30 days postnatally (P1–P30). The experiments were performed in accordance with Directive 86/609/ CEE of the European Community Council, the Spanish Real Decreto 223/1988, and Seville University regulations on laboratory animal care.

**Electrophysiological identification and injection of the Mns**

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg) and quickly decapitated. The method to obtain the slices is fully detailed elsewhere (Carrascal et al., 2006). In brief, slices (400 μm) including the oculomotor nucleus were firstly incubated in a chamber containing cold sucrose-artificial cerebrospinal fluid (ACSF) for 35–45 minutes and then transferred to a second chamber containing ACSF at a temperature of 2°C ± 1°C. Single slices were finally transferred to the recording chamber and superfused at 2 ml/minute (Harvard MPII) with ACSF bubbled with 95% O2-5% CO2 (pH 7.4: 21°C ± 1°C). The composition of ACSF was as follows (in mM): 126 NaCl, 2 KCl, 1.25 Na2HPO4, 26 NaHCO3, 10 glucose, 2 MgSO4, and 2 CaCl2. For sucrose/ACSF solution, the 126 NaCl was substituted by 240 mM sucrose.

All recorded and labeled neurons were identified as Mns by their antidromic activation from the root of the third nerve and by the collision test (for details see Fig. 1 in Carrascal et al., 2006). Micropipettes for intracellular injection were filled with 3 M KCl (tip resistance 60–100 MΩ) containing 2% neurobiotin (Vector Laboratories, Burlingame, CA). Neurobiotin was iontophoresed into the Mns by passing a depolarizing current pulse (0.2–1 nA) for a duration of 500 msec at 1 Hz. Care was taken to impale only a single cell per slice from the oculomotor Mn pool. The duration of iontophoresis ranged from 15 to 60 minutes.

After intracellular injection of neurobiotin, the slices were incubated in normal ACSF for 1–4 hours and then immersed in fixative (4% paraformaldehyde) at 4°C for 1–2 days. Next, slices were moved to 30% sucrose in phosphate buffer (0.05 M; pH 7.4) at 4°C overnight. On the following day, these slices were washed in 0.05 M Tris-buffered saline (TBS; pH 7.4), and the endogenous peroxidase activity was suppressed by incubating slices in 5% H2O2 in 0.05 M TBS and methanol for 15 minutes, followed by washing in 0.05 M TBS for 15 minutes. Then, the slices were pretreated with 1% Triton X-100 for 2–3 hours and incubated with an avidin-biotin-peroxidase complex (Vectorstain Elite; Vector Laboratories) diluted in 0.05 M TBS and 0.3% Triton X-100 (TBS+, pH 8.6) for 3 hours at room temperature. After being rinsed with TBS+ (15 minutes) and a 0.05 M Tris-HCl (pH 7.6) buffer (15 minutes), slices were reacted with ammonium nickel sulfate (1%) and 3’3’-diaminobenzidine solution (DAB) and washed in Tris-HCl (2 × 10−2 M; pH 7.4).
15 minutes). Slices were mounted on gelatinized slides, dehydrated with serial alcohols, cleared with xylene, and coverslipped with DPX (Fluka, Buchs, Switzerland).

The slice shrinkage was measured in the X, Y, and Z axes. Shrinkage in the X-Y axes was similar and was lower than 9%. Therefore, no corrections were applied in these directions. The shrinkage in the thickness (Z plane) was evaluated by measuring the average Z-distance between the top and the bottom of the slice with a laser-scanning confocal microscope (Leica TCS-SP2) and dividing by the nominal thickness (400 μm). The shrinkage factors ranged between 0.2 and 0.4. The greatest shrinkage was found in the slices from the youngest animals. Z-axis corrections were applied for each neuron on the basis of its individual factor.

**Neuronal reconstruction and morphometric analysis**

For morphological analysis, Mns were first reconstructed in 3D using the Neuroulcida system (MicroBrightField, Williston, VT) on an Olympus microscope (BX61; Olympus, Tokyo, Japan) at ×63 magnification. Morphometric measurements were extracted from the reconstruction files in Neuroexplorer software (MicroBrightField). The photomicrographs were taken, using the Nomarski technique, with a digital camera coupled to the microscope (DP70; Olympus) at ×20, ×40, ×100 and were processed in Adobe Photoshop 7.0 (Adobe System Inc., San Jose, CA) to adjust contrast and brightness. The data for the analysis were plotted and quantified in Origin software 7.5 (Originlab Corporation, Northampton, MA).

Reconstructed Mns met the selection criteria followed elsewhere (Nunez-Abades et al., 1994). First, Mns had a healthy appearance, with no evidence of impalement damage to the somal membrane, minimal extracellular dye leakage, and a good contrast with low background labeling of the tissue from the ABC reaction. The dendritic arborization was contained within the slices, because terminations corresponded to natural endings (final diameter ranged between 0.2 and 1 μm) as opposed to cut branches. Among the 135 Mns injected with neurobiotin, only 76 showed good labeling. Fifty of these well-stained Mns were fully reconstructed, based on the criteria indicated above, and grouped as follows: 40 Mns were multipolar, five were bipolar, and five were coupled to a neighboring cell. Morphometric analyses were restricted to the 40 isolated multipolar Mns.

Figure 1 illustrates the computer-assisted reconstruction of a P4 Mn (Fig. 1A) and the dendrogram of its six dendrites (Fig. 1B,C). Each dendritic branch was assigned a branch order (following a centrifugal ordering method: from cell body to terminal branch), and each branch was identified as a preterminal or a terminal branch (Fig. 1C). The term *preterminal branch* was used to identify parts of a dendrite that connected the soma with the first branching point or two successive branching points. A terminal branch was defined as a branch that connected a branching point with a terminal ending.

The quantitative data of morphometric parameters reported here were provided by Neuroexplorer software. The somal perimeter was outlined to measure the somal membrane area. The dendrites were reconstructed by making multiple measurements (approximately every 5–10 μm), assigning X, Y, Z, and diameter values. The dendritic mean diameter was the average diameter of all first-order dendrite branches of a neuron. To measure dendritic complexity, the branch order was scored calculated as the average of the order of all branches, and the terminal order as the average of the order of terminal branches. The combined dendritic length was calculated as the summed lengths of all branches of a neuron; the mean branch length was the average between all the branches of a neuron, which included preterminal and terminal branches, and the terminal branch length was the average of the length of all the terminal branches of a dendrite. A Sholl diagram was generated for all reconstructed Mns (Sholl, 1953). To build these plots, the Mns were oriented in the same
dorsoventral and mediolateral axes, and then a composite drawing of 10 Mns corresponding to each age group was made. The Sholl diagram measured the location of dendrites as a function of the distance to the soma, plotting the radial distance crossed by dendritic branches. Thus, the somata of each composite drawing were placed in the center of circles whose radius increased in successive steps of (in our study) 25 μm, and the number of dendrites intersecting each circle was scored.

Statistical analysis

All statistical analyses were carried out on the raw data. Mns were classified into four postnatal age groups (P1–P5, P6–P10, P11–P15, and P21–P30). The number of Mns per age group was 10. Taking together the data of all age groups for each parameter, first we determined whether there were significant differences along postnatal development, by using a one-way ANOVA test. If there were significant differences, then a parametric Tukey test between adjacent age groups was applied (i.e., first vs. second age groups; second vs. third age groups, etc.). We established the significance level at \( P < 0.05 \), indicated in the tables by an asterisk. The Tukey test was also applied to compare the first and fourth age groups. In this latter case, a similar criterion of significance was used, indicated in the tables by a dagger.

RESULTS

Morphological features

Figure 2 illustrates three representative labeled oculomotor nucleus Mns. The most frequent Mns (65 of a total of 76, i.e., 85%) showed polygonal cell bodies, with four to eight primary dendrites (five on average) emerging from different somal poles. These Mns were termed multipolar (Fig. 2A–D, Table 1). Inspection of these Mns indicated that the dendritic spatial distribution depended on their location in the nucleus. The Mns near the center of the nucleus showed dendrites with radial spreading (Fig. 2A,B), whereas those close to the edge of the nucleus had dendritic trees preferentially oriented toward the inner aspects of the nucleus (Fig. 2C,D). Mns with fusiform cell bodies were occasionally found (11 of a total of 76, i.e., 15%); they displayed two to four primary dendrites emerging from the main somal axis and were termed bipolar (Fig. 2E,F). These latter Mns were not found either at any particular location within the nucleus or at any particular postnatal age. All bipolar Mns had dendritic branches that generally exited the nucleus; it was possible to follow them for up to 1 mm from the soma (Fig. 2F). In contrast, the vast majority of dendritic arborizations of multipolar Mns were confined within the oculomotor nucleus. The dendritic branches leaving the OCM nucleus were found in all directions but were chiefly oriented to the dorsal and ventrolateral areas. Four multipolar Mns showed dendrites crossing the midline and running into the contralateral oculomotor nucleus.

Morphometric analyses

The increase in the oculomotor nucleus size and motoneuronal dendritic trees with postnatal development is illustrated in Figure 3 and quantified in Table 1. The nucleus size (mean diameter = largest + smallest diameters/2) increased continuously with age from 313.1 μm (P1–P5) to 402.4 μm (P21–P30), i.e., a mean increase of 80 μm. The maximum dendritic length, defined as the distance from the soma to its farthest terminal ending in any direction, also grew with age from 458.9 μm (P1–P5) to 729.5 μm (P21–P30), i.e., an increase of 270.6 μm. The fact that the increase in the maximum dendritic length was greater than that of nucleus size with maturation demonstrated that increasing length of the dendrites was not exclusively a consequence of increasing nucleus size. Although Mns of all age groups have dendrites with some dendritic terminals lying outside the boundaries of the nucleus, the area of membrane located outside increased about four-fold with age: 1,420.8 μm² (P1–P5) vs. 5,823.1 μm² (P21–P30). Therefore, the dendritic membrane located outside the nucleus represented 15.4% of the total membrane area of dendritic trees in newborn animals (P1–P10) and 25.2% in the oldest age group (P21–P30).

The arborization pattern of oculomotor nucleus Mns was altered with maturation. The number of dendritic trees per neuron was five on average at birth, and this number did not change with the course of postnatal development, but the dendritic architecture was largely remodeled in this period. Figure 4 plots the raw data (each open circle corresponds to a single Mn) and mean value (solid circles) for each age group of the different parameters studied to measure the dendritic complexity. Two phases were distinguished in the process of dendritic shaping. After birth, the number of terminals per neuron increased from 26.3 (P1–P5) to 47.7 (P6–P10). Later, the number of terminals fell significantly to 30.6 (P21–P30; Fig. 4A, Table 2). The biphasic nature of dendritic shaping was also corroborated by the variation in the number of branches, terminal order, and branch order (Fig. 4B–D, Table 2). Thus, we found an initial increase in the number of dendritic branches (57.2 P1–P5 vs. 93.9 P6–P10), terminal order (3.5 P1–P5 vs. 4.4 P6–P10), and branch order (2.8 P1–P5 vs. 3.8 P6–P10), followed by a simplification of the arborization at P11–P15. In the adult rats, the values for these parameters were similar to those scored at birth. These findings revealed that changes in the number of branches, terminal order, and branch order coursed in parallel to the changes in the number of dendritic terminals with age. All these data together demonstrated that the dendritic complexity of Mns increased after birth (reaching a peak value by about P8) and then simplified, showing characteristics similar to those of newborn subjects. Postnatal development also produced alterations in dendritic length. Figure 5A–D illustrates composite drawings of 10 reconstructed Mns, corresponding to each postnatal age group, that were quantified by the Sholl diagrams (Fig. 5E–J). In the first age group (P1–P5), it was found that the maximum dendritic intersection extended to 350 μm, and the peak number of dendritic intersections was 150 (Fig. 5E). In the second age group (P6–P10), the maximum dendritic intersection extended to 400 μm, and the number of intersections was 225 (Fig. 5F). In the age group between P11 and P15, the maximum dendritic intersection reached 500 μm and the dendritic intersections 160 (Fig. 5G). In the oldest age group studied (P21–P30), the maximum dendritic intersection extended to 700 μm, and the peak number of intersections was 125 (Fig. 5H). Figure 5I illustrates a cumulative plot of the number of dendrites that intersected each radial distance for the different age groups. As can be seen, the main difference was that the greatest dendritic complexity was found between P6 and P10, the total number of dendritic intersections being about...
Figure 2.
Representative morphologies of oculomotor nucleus Mns. A,C,E: Photomicrographs of multipolar (A,C) and bipolar (E) intracellularly labeled Mns. B,D,F: Reconstruction of the labeled Mns illustrated in photomicrographs. Dots indicate the location of the somata within the nucleus of reconstructed Mns, and stars illustrate the location of the cell depicted. Note that multipolar Mns display dendrites oriented radially (B) or toward the inner aspects of the nucleus (D), depending of the somal location. Axons have been omitted from this and the following figures. Scale bars = 50 μm in A,C,E; 100 μm in B,D,F.
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1,600 in this period, whereas in the other age groups it was lower (in newborn Mns about 1,000, and in adult Mns about 1,300). In other words, there was a transient increase in complexity in the epoch P6–P10. In addition, we plotted the data of Sholl diagrams in a cumulative probability plot (normalized by the maximum number of cumulative dendritic intersections; Fig. 5J). This plot demonstrates that the dendrites tended to elongate with age; it was found that most of the dendrites (75%) extended in radial distance about 170 μm (P1–P10), increasing to 200 μm (P11–15) and then to 260 μm (P21–P30). Furthermore, the increase in complexity between P6 and P10 did not involve elongation (in relation to the length of newborn Ms), insofar as most of the dendrites (75%) extended for to a similar radial distance along these postnatal days. These latter results suggest that the transient increase in dendritic complexity may result from a sprouting of new terminal branches over the whole order of dendritic tree branches.

The temporal course of the dendritic elongation is illustrated in Figure 6 and quantified in Tables 1 and 2. Figure 6A plots the maximum distance from the somata to dendritic terminal endings with age. In the youngest Mns (P1–P5), the maximum distance ranged between 300 and 550 μm; in the oldest Mns (P21–P30), it was between 500 and 1,000 μm. The increase in the maximum dendritic length during postnatal development was linearly fitted (P < 0.001; r = 0.6), showing a slope of 14.02 μm/day. To determine how elongation occurred with age, in Figure 6B is plotted the combined dendritic length of the tree of individual cells against postnatal age. The best fit to the raw data was a single exponential curve (r = 0.5, τ = 5.7 days). The time constant of this fit showed that the combined dendritic length rose sharply early during postnatal development, in that 63% of the total increase was in the first 5 days after birth. The early increase in the combined dendritic length was also supported by the differences found between P1–P5 and P6–P10 (Table 2).

TABLE 1. Comparison of the Growth of the Oculomotor Nucleus and Motoneuronal Dendritic Trees During Postnatal Development

<table>
<thead>
<tr>
<th>Variable</th>
<th>Age group (P)</th>
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<tbody>
<tr>
<td></td>
<td>1-5</td>
</tr>
<tr>
<td>Medium diameter of nuclei (μm)</td>
<td>313.1 ± 9.4</td>
</tr>
<tr>
<td>Max. dist. soma-terminal ending/neuron (μm)</td>
<td>458.9 ± 24.6</td>
</tr>
<tr>
<td>Area of dendrites outside nuclei (μm²)</td>
<td>1,420.8 ± 313.6</td>
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<tr>
<td>Percentage of dendrites outside nuclei</td>
<td>15.4 ± 2.5</td>
</tr>
</tbody>
</table>

†P < 0.05 between first and last age groups (Tukey test).

To assess the contribution of terminal branches or all branches (without differentiating between terminal and pre-terminal branches) to dendritic elongation, two dendritic branch parameters were analyzed separately. Figure 6C shows that there was a transient decrease in the length of terminal branches between 6 and 10 postnatal days (P1–P5 = 125.3 μm vs. P6–P10 = 82.4 μm); later in development, the length of these dendritic segments was similar to that measured at birth. Taking together the data from the Sholl diagram, indicating an increase in dendritic complexity for the period between P6 and P10, and the increase in the number of terminal branches scored in this period (Fig. 4A), it is plausible that the transient decrease in dendritic terminal length is the result of new terminals sprouting rather than a resorption of distal branches. Furthermore, the changes in the length of all branches showed a different temporal sequence (Fig. 6D). Thus, whereas the scored data did not reveal any difference below P10, the dendritic branch length increased later, with significant differences between the youngest and the oldest age groups (P1–P10 = 85.3 μm vs. P21–P30 = 116.1 μm).

Finally, we studied the changes in the diameter and area of dendrites with age, as well as the modifications in the somal surface (Fig. 7, Table 2). The diameters of the first-order dendrites for each age group increased with age, the raw data fitting a single exponential (r = 0.5, τ = 7 days; Fig. 7A). The increase in diameter of first-order dendrites was found within a short time after birth (below P10), and the statistical analysis showed significant differences between the youngest age group (P1–P5 = 2.2 μm) and P6–P10 (2.9 μm). Then, there was a slow gradual increase in diameter (Table 2). Figure 7B depicts the increase in the total dendritic membrane area with maturation. The raw data also fitted a single exponential (r = 0.62; τ = 8.8 days); i.e., 63% of the total increase of dendritic size was achieved in about 9 postnatal days. There were significant differences in the dendritic area between age groups P1–P5 (10,076.9 μm²) and P6–P10 (17,871.1 μm²). Later, there was a slow, gradual increase in dendritic membrane area (Table 2). Therefore, the temporal course leading to the increased diameter of the first-order dendrites and total dendritic area was essentially the same, and the largest diameters involved the largest dendritic membrane area. Similar conclusions have been reached for other motoneuronal pools; i.e., these correlations could be a general principle governing the growth and maintenance of the dendrites of mammalian Mns (Nunez-Abades and Cameron, 1995).

The somal membrane area also increased with age (Fig. 7C, Table 2). In fact, the raw data fitted a single exponential (r = 0.58; τ = 10.3 days), and statistical differences were found when the first (P1–P6 = 1,040.2 μm²) and third (P11–P15 = 1,718.3 μm²) age groups were compared. In other words, somal size was not established at birth, but the adult somal size was achieved by about P10–P15. Furthermore, the increase in somal membrane area during postnatal development was smaller than the increase in dendritic membrane area. The somal area was 1.65-fold larger in the adult than in newborn subjects, whereas the dendritic membrane area increased by a factor of 2.25. With regard to the temporal course, the time constants indicated that the greatest increase in dendritic area took place in an epoch similar to that in somal area. However, the latter was achieved by P15, while the dendritic area continued to increase slowly. The total area (somatic + dendritic) increased exponentially with age (r = 0.64; τ = 9 days; Fig. 7D). The dendritic area represented some 90% of the total area at birth and 94% in adults (Table 2). Thus, the main increase took place between P1–P5.
(11,289.9 \mu m^2) and P6–P10 (19,235.8 \mu m^2), with a subsequent slow growth up to P21–30 (24,700.3 \mu m^2).

**Tracer coupling**

In five cases (all restricted to rats younger than 9 days old, representing five cases among a total 23 labeled Mns in this period), tracer coupling was observed. The photomicrograph in Figure 8A illustrates in a P3 animal a representative example of two intensely labeled coupled cells within the oculomotor nucleus. The tracer-coupled neurons were distributed as follows: somatosomatic (n = 2), somatoprimary dendritic coupling (n = 2), dendrodendritic (n = 1). In the latter case, the somata were separated by 80 \mu m, but both were lying inside the nucleus. Irrespective of the type of coupling, the neurobiotin spread was restricted to one additional cell. No examples were observed of Mns coupled across the midline, belonging to the two oculomotor nuclei. The observation and reconstruction (of labeled somata and dendrites) revealed that, in morphological terms, the coupled Mns were similar to and indistinguishable from those termed here multipolar (Fig. 8A,B). A preferred location of the coupled cells was not observed (Fig. 8C).

**DISCUSSION**

The present study reports for the first time the temporal sequence of changes in dendritic morphometry of rat oculomotor nucleus Mns during postnatal development. The maturation process can be summarized in two phases. During the first phase (from birth to about P10), the membrane area of dendritic trees increased by intensification of the degree of arborization. In this period, there was also an increase in somal size. Occasionally, coupled Mns were observed. During the second phase (beyond P10), maturation produced a greater area farther from the soma, and the complexity of the dendritic arborization decreased. These results will be discussed in relation to the different proposed patterns of postnatal development in other motoneuronal pools and in the context of maturation of the electrophysiological properties and oculomotor neural circuits. First, we will compare the morphology of the labeled Mns found here with that reported in rat and other species, technical restrictions of the method, and rate and functional meaning of coupling.

**Morphology of the ocular Mns and technical considerations**

Intracellular injections of tracers, in acute preparation, have been carried out in different mammalian species (Grantyn and Grantyn, 1978; Highstein et al., 1982; McCrea et al., 1986; Russell-Mergenthal et al., 1986; Evinger et al., 1987), including the rat (Durand, 1989a,b). According to the somatodendritic shape, the Mns are similar across the different studied species (for review see Evinger, 1988). In adult rats, 10 oculomotor neurons were intracellularly labeled with horseradish peroxidase and fully reconstructed (Durand, 1989b). The somal size, the number of first-order dendrites, and the territory in which dendrite arborization extended were similar to the data reported here for multipolar Mns in an in vitro preparation. Although multipolar Mns were observed more frequently (85%, 65/76), bipolar Mns were also found occasionally (15%, 11/76). Irrespective of the postnatal age, bipolar Mns had smaller somata than multipolar Mns, with larger dendrites that
Figure 4.
Changes in dendritic complexity in oculomotor nucleus Mns during postnatal development. Scatterplot showing the changes in the number of terminal branches (A), number of branches (B), terminal order (C), and branch order (D) as a function of postnatal age. Open circles indicate data from individual Mns, and solid circles are the averages of pooled data in the different age groups. Solid lines connect solid symbols.

TABLE 2. Somatodendritic Morphometry of Oculomotor Nucleus Mns During Postnatal Development

<table>
<thead>
<tr>
<th>Variable</th>
<th>1-5</th>
<th>6-10</th>
<th>10-15</th>
<th>21-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary dendrites/neuron</td>
<td>5.0 ± 0.2</td>
<td>5.4 ± 0.4</td>
<td>5.1 ± 0.4</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>Terminal branches/neuron</td>
<td>26.3 ± 3.8</td>
<td>47.7 ± 7.8</td>
<td>30.0 ± 1.2*</td>
<td>30.6 ± 4.1</td>
</tr>
<tr>
<td>Branches/neuron</td>
<td>57.2 ± 11.2</td>
<td>93.9 ± 16.5*</td>
<td>49.0 ± 5.3*</td>
<td>43.5 ± 4.2</td>
</tr>
<tr>
<td>Terminal order</td>
<td>3.5 ± 0.3</td>
<td>4.4 ± 0.3*</td>
<td>3.8 ± 0.1</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Branch order</td>
<td>2.8 ± 0.2</td>
<td>3.8 ± 0.2*</td>
<td>3.4 ± 0.1</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Combined dendritic length (μm)</td>
<td>4,287.9 ± 613.9</td>
<td>5,756.1 ± 551.5</td>
<td>5,579.3 ± 337.2</td>
<td>6,462.2 ± 240.4†</td>
</tr>
<tr>
<td>Terminal dendritic length (μm)</td>
<td>125.3 ± 10.5</td>
<td>82.4 ± 8.5*</td>
<td>122.0 ± 5.7*</td>
<td>123.5 ± 7.8</td>
</tr>
<tr>
<td>Branch dendritic length (μm)</td>
<td>85.3 ± 6.4</td>
<td>86.5 ± 8.5</td>
<td>105.3 ± 4.3</td>
<td>116.1 ± 8.8†</td>
</tr>
<tr>
<td>Diameter of primary dendrites/neuron (μm)</td>
<td>2.20 ± 0.2</td>
<td>2.86 ± 0.1*</td>
<td>3.26 ± 0.1</td>
<td>3.39 ± 0.2†</td>
</tr>
<tr>
<td>Dendritic area/neuron (μm²)</td>
<td>10,076.9 ± 1,490.9</td>
<td>17,671.1 ± 2,432.2*</td>
<td>18,691.2 ± 2,178.5</td>
<td>22,973.4 ± 1,289.9†</td>
</tr>
<tr>
<td>Somatic area (μm²)</td>
<td>1,040.2 ± 113.7</td>
<td>1,208.7 ± 123.1</td>
<td>1,718.3 ± 115.5</td>
<td>1,726.9 ± 125.8†</td>
</tr>
<tr>
<td>Total area/neuron (μm²)</td>
<td>11,117.2 ± 1,538.8</td>
<td>19,235.8 ± 2,224.9*</td>
<td>20,409.6 ± 2,209.1</td>
<td>24,700.3 ± 1,301.7†</td>
</tr>
</tbody>
</table>

*P < 0.05 between adjacent age groups (Tukey test).
†P < 0.05 between first and last age groups (Tukey test).
exited the oculomotor nucleus limits (for comparison see Fig. 2). According to their proportion, somata sizes, and dendritic length, bipolar and multipolar Mns could correspond to those termed elsewhere as multiply innervated muscle Mns and singly innervated muscle Mns, respectively (Eberhorn et al., 2006; Horn et al., 2008).

Several concerns are inherent to the intracellular labeling, reconstruction, and quantification in slice preparations. First, cell impalement using sharp electrodes is less difficult in larger Mns. Therefore, the probability that the sample of injected Mns was skewed toward Mns of greater size cannot be ruled out, but we were able to label in the newborn animal Mns of about half the size of those in the adult, leading to the conclusion that the microelectrodes were not particularly selective. Second, the neurobiotin tracer was not effectively transported to label the whole dendritic tree. We assume that our data were not skewed by the tracer transport, because we selected the Mns showing labeled dendritic terminal branches with diameters of less than 1 μm (Nunez-Abades et al., 1994). In addition, the full reconstruction of adult Mns revealed dendritic territories about twofold larger than those in the newborn, making it plausible that dendritic labeling during the postnatal period was not underestimated. On the other hand, the rat Edinger-Westphal nucleus is situated ventral to cerebral aqueduct, just lateral to the midline, and mainly rostral to the oculomotor nucleus (Klooster et al., 1993). The Edinger-Westphal preganglionic parasympathetic neurons exit via the third cranial nerve innervating to the ciliary ganglion, thereby controlling pupillary constriction and lens accommodation (Gamlin et al., 1994; Vilupuru and Glasser, 2002). Thus, it is possible that, in using the antidromic activation and collisions tests to identify the injected cells, some of the Edinger-Westphal preganglionic parasympathetic neurons were not identified.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Spatial distribution of dendrites in oculomotor nucleus Mns for the different age groups. A–D: Composite drawings of 10 Mns corresponding to each age group. E–H: Sholl diagrams illustrating the total number of intersections of dendrites, corresponding to each age group, with a series of concentric spheres centered within the cell body and placed at 25-μm interval increments. I: Plot of cumulative dendritic intersections of the data shown in E–H relative to the radial distance to the soma. J: Plot from the cumulative diagram (I), illustrating the probability of dendritic intersections, normalized by the maximum cumulative dendritic intersection. Scale bar = 100 μm.
Wesphalt neurons were included here as Mns. We assume that all analyzed multipolar cells were Mns and not preganglionic neurons based on two criteria: labeled cells were placed inside the oculomotor nucleus limit; i.e., they were not situated within Edinger-Westphal nucleus boundaries (Klooster et al., 1993); and the reported somata of the preganglionic Edinger-Wesphalt neurons (Laursen and Rekling, 2006) are smaller than those of the labeled cells reported here.

**Coupling and postnatal development**

The present work shows that, early in postnatal development, some Mns are tracer coupled, which supports the idea of the presence of gap junctions. A similar finding has been reported for spinal Mns (Walton and Navarrete, 1991; Martin-Caraballo and Greer, 1999). It has been proposed for genioglossal Mns that coupling could serve to synchronize their collective discharge, producing a more robust and uniform protrusion of the tongue required for several motor tasks, such as sucking, breathing, and swallowing, as soon as the animal is born (Mazza et al., 1992). The hypothesis that electrotonic coupling in the postnatal period could subserve motor functions is difficult to extend to ocular Mns, because they are not ready to work until P21, when the eye movements evoked by visual and vestibular stimuli are performed (Lanou et al., 1980; Faulstich et al., 2004). In any case, the loss of electrotonic coupling with age in Mns would release control of muscle fibers to individual motor units (Walton and Navarrete, 1991). Alternatively, neuronal coupling during the first 2 postnatal weeks could serve to set up a “prewiring” that helps a progressive spatial and temporal formation of neural circuits (Montoro and Yuste, 2004; Szabo and Zoran, 2007).

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**Figure 6.** Changes in dendritic length in oculomotor nucleus Mns during postnatal development. Scatterplot showing the changes in maximum distance from soma to terminal ending (A), combined dendritic length (B), terminal branch length (C), and branch length (D) as function of postnatal age. Open circles indicate data from individual Mns; solid circles are the averages of pooled data in the different age groups. Dashed lines are the best fits to the raw data in A, B, and solid lines connect solid symbols in C, D.
Somatodendritic morphometric changes during postnatal development

The sample of labeled oculomotor nucleus Mns reported here included four different postnatal age groups (P1–P5, P6–P10, P11–P15, and P21–P30). The duration in days for the age groups was similar to that reported for hypoglossal nucleus and spinal Mns (Nunez-Abades et al., 1993, 1994; Viana et al., 1994; Li et al., 2005). Furthermore, during the first 2 postnatal weeks, there are significant changes in input resistance and time constant in oculomotor nucleus Mns (Carrascal et al., 2006) that may reflect somatodendritic morphometric changes. In short, we focused on age groups up to P15 and then comparison with young adult rats (P21–P30).

Dendrite morphogenesis is a highly dynamic and regulated process that involves the formation of new branches as well as the maintenance or elimination of preexisting ones (Jan and Jan, 2003; Chen and Ghosh, 2005; Ye and Jan, 2005). It has been proposed that, during developmental periods of synaptogenesis, synaptic activity increases the emergence of fine dendritic branches (Cline, 2001). The study of the complexity of dendritic trees in the oculomotor nucleus Mns allows the establishment of two phases in the maturational process. During the first phase (up to P10), the number of first-order dendrites did not change, but the number of branches increased, leading to a progressive increase in the complexity. During the second phase, the dendritic arborization decreased until reaching values of complexity similar to those in newborn Mns. A similar pattern has been described for retina ganglion cells (Coombs et al., 2007). Studies conducted in spinal lumbar Mns have demonstrated a different

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Figure 7.
Changes in dendritic size in oculomotor Mns during postnatal development. Scatterplot showing the changes in diameter of first-order dendrites (A), dendritic area (B), somal area (C), and total area (D) as a function of postnatal age. Open circles indicate data from individual Mns; solid circles are the averages of pooled data in the different age groups. Dashed lines are the best fits to the raw data.
Enlargement of neonatal mouse lumbar Mns during the first 2 weeks of postnatal development was comparable to the growth of the spinal cord. However, in oculomotor nucleus Mns, the increase in length did not equal the increase in size of the nucleus; as a result, dendrites of newborn Mns extended outside the nucleus in a smaller area and percentage than those of adult Mns. In the transverse section, the dendrites of adult Mns were located outside the nucleus preferentially oriented dorsally and ventrolaterally. It would be suitable that preference in dendritic elongation was promoted by the source of afferents, as demonstrated in other neuronal pools (Cline, 2001). Thus, dendrites ventrally oriented tended to be closer to the location of the medial longitudinal fasciculus (the source of vestibular afferents that start to drive eye movements at about P21) and correlate well temporally with the dendritic elongation of their second phase. The extension of the dendrites of both flexor and extensor Mns in rat cervical spinal cord changed, from spreading in all directions on postnatal day 2 to spreading in only a few, specific directions from postnatal day 21 onward (Curfs et al., 1993). For genioglossal Mns, dendrites exceeding the boundary of the nucleus have also been reported, particularly in the adult epoch, and it has been proposed that dendrites in these new territories are related to the increasing importance of the input received in those sectors during development (Nunez-Abades et al., 1994). When the signals triggering dendritic enlargement and the molecular events underlying this process become well understood, it would be of great interest to determine how dendritic growth is regulated temporally (Zhang and Huang, 2006).

We have found that major changes in somal area and dendritic area course in parallel during the first 2 postnatal weeks. This is when the adult somal size was achieved, whereas dendritic membrane area continued to increase slowly up to P30. The ratio of dendritic area to somal area increases postnatally in spinal and genioglossal Mns (Ulfhake and Cullheim, 1988; Cameron et al., 1991). Major differences have been reported in rat genioglossal Mns, in which the somal size is established at birth, whereas the dendritic membrane surface increases during at least 1 month. These data indicate that signal(s) promoting the increase in membrane surface area is different for the somata and dendrites and/or that the response is different.

**Relationship between somatodendritic area and passive membrane properties during postnatal development**

With postnatal development, the membrane surface area of the soma and dendrites in oculomotor nucleus Mns increased exponentially, with a similar time constant, until about P8–P10. Most of the increases in these parameters occurred during the first 2 weeks. A primary goal of the present study was to determine whether the already reported modifications in passive membrane electrophysiological properties of oculomotor nucleus Mns (Carrascal et al., 2005, 2006) could be due to changes in the total somatodendritic membrane area.
as previously demonstrated for Purkinje cells (McKay and Turner, 2005). In oculomotor nucleus Mns, the input resistance declines sharply early during postnatal development (P1–P5). This decrease follows a single exponential decay, with a time constant of 3.1 postnatal days. The mean input resistance in newborn Mns was 82.9 MΩ, and in adult Mns 53.1 MΩ. The membrane time constant also decreased exponentially (time constant = 3.9 postnatal days), with a temporal course similar to that of input resistance: 9.4 msec in the newborn Mns and 4.9 msec in adult Mns. We have previously demonstrated a significant negative linear regression between input resistance and the somatodendritic area in adult Mns (Nieto-Gonzalez et al., 2007). In broad terms, the increase in total somatodendritic membrane surface area reported here in oculomotor nucleus Mns could underlie the decrease in input resistance and time constant previously reported. However, the exponential time constants of electrophysiological parameters (about 3–4 days postnatally) and morphological measurements (about 8–10 days old) support the notion that the two are not completely linked. These differences suggest that other mechanisms should be added to explain the sharp decrease in passive membrane properties during early development.

In contrast to oculomotor nucleus Mns, phrenic and genioglossal Mns show postnatal periods in which the total surface area does not change: younger than 2 weeks in rat genioglossal Mns and 1 month in cat phrenic Mns (Cameron et al., 1991; Nunez-Abades and Cameron, 1995). In these motoneuronal pools, a decrease in input resistance and time constant up to the first postnatal month for the cat phrenic Mns and up to P15 in rat genioglossal Mns has also been reported. In these periods the total membrane surface area does not increase, so it was proposed that the installation of leak channels could underlie the reduction in passive membrane properties (Nunez-Abades et al., 1993; Cameron and Nunez-Abades, 2000). This proposal is supported by studies showing that the blockage of the potassium conductances increases the input resistance in adult Mns more than in newborn ones (Cameron et al., 2000; Nunez-Abades et al., 2000). According to these findings, determining the contribution of the leak potassium channels to the reduction in passive membrane properties during early postnatal development in oculomotor nucleus Mns would require further research.

Temporal sequence of morphometric changes for oculomotor nucleus Mns in the context of the oculomotor system maturation

The generation of dendritic trees is required for establishment of precisely wired neuronal networks (Scott and Luo, 2001; Jan and Jan, 2003). Environmental cues and intrinsic genetic factors are thought to act jointly to regulate dendritic growth and remodeling (Steljes et al., 1999; van Aelst and Cline, 2004; Wu et al., 2007). Trophic factors, provided by afferents and/or target, and/or the installation or removal of synapses could contribute to changes in somatodendritic remodeling (Steljes et al., 1999; Mertz et al., 2000; Cline, 2001). With regard to the inputs, oculomotor nucleus Mn activity is essentially triggered by vestibular and visual sensory signals (Büttner-Ennever, 2005). We have found that, during the first phase of Mns maturation, there was a major increase in somatodendritic area and dendritic complexity (up to 10 postnatal days). In this period, somatic and dendritic growth occurs in vestibular neurons (Puyal et al., 2002) and is primarily building the circuitry transmitting the signal from the vestibular nuclei to the ocular Mns (Curthoys, 1981; Rusch et al., 1998), and the electrical stimulation of the vestibular nerve evokes eye movement (Curthoys, 1979). The second maturational phase reported here for oculomotor nucleus Mns was later than P10 with elongation of dendrites. This period is established at the time when the eyelids open. Eye movements evoked by visual and vestibular stimuli are performed from about P21 in rodents, although the efficacy of these reflexes increases a few days later, allowing clear vision during self-motion (Lannou et al., 1980; Faulstich et al., 2004). With regard to the target, changes in myelination, neuromuscular junctions, and muscle elements occur during different postnatal periods of extraocular muscle morphogenesis (Porter and Karathanasis, 1999; Cheng et al., 2004). Which muscle, vestibular, and visual trophic signals arriving at the oculomotor nucleus, and/or installation-removal of synapses, contribute to the differentiation of the Mns, and how they act during the two maturational periods to shape oculomotor nucleus Mns, are exciting questions to investigate in the near future.

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LITERATURE CITED


Curthoys IS. 1981. The organization of the horizontal semicircular duct,


