Research Articles

Role of extracellular matrix molecules in the development of the sodium current in quail mesencephalic neural crest cells

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Abstract. The occurrence of the voltage-dependent sodium current has been studied in developing neurons from quail mesencephalic neural crest on different substrates, using the whole-cell patch clamp technique. Explants from 9–12 somite embryos were cultured on dishes coated with type I collagen, fibronectin, laminin or on plastic dishes in a chemically defined medium. After 18 h of culture the sodium current was observed in 70% of the neurons tested, and at 24 h some of these neurons were able to generate an action potential. After 18–25 h cells grown on fibronectin- or collagen I-coated dishes showed a significantly higher occurrence of the sodium current (83% and 84% respectively) as compared to cells grown on uncoated plastic dishes (51%). Moreover, in the presence of fibronectin, the current density of the sodium current was more than doubled in comparison with cells grown on other substrates.
Key words. Quail mesencephalic neural crest; electrophysiology; sodium current; fibronectin; laminin; collagen I; development.

During embryonic development, environmental interactions are crucial in modulating a variety of cell responses, such as growth, migration and differentiation [3, 5], and in determining the final fate of the precursor cells [12]. An important role appears to be played by the interactions between the cell surface and extracellular substrates [16, 20]. In particular, during the development of peripheral neurons, two components of the extracellular matrix (ECM), fibronectin (FN) and laminin (LN), are able to promote neurite outgrowth in neurons from embryonic dorsal root (DRG) and sympathetic ganglia [17]. Moreover, the presence of at least one of these glycoproteins is necessary for the in vitro differentiation of neuronal precursor cells isolated from chick DRG [8].

FN and type I collagen (COLL) are the major components of the ECM in the cranial pathways of neural crest migration [15]; in vivo the neuronal precursors are exposed to FN and express receptors for this molecule early in development [6], and it has been proposed that FN, through its own adhesive properties and its ability to bind collagens and other ECM molecules, may promote some aspects of neuronal differentiation [19]. On the other hand, LN is reported to be involved in the later stage of neural crest cell aggregation into ganglia [7].

The aim of the present study was to investigate, using the whole-cell patch clamp technique, the different roles of these three ECM molecules in the development of the electrical membrane properties in differentiating neurons from quail mesencephalic neural crest (MNC) explants. In MNC neurons [1, 2], as in other neuronal populations [9, 13, 22, 23], the various currents do not appear simultaneously, but follow a specific temporal sequence. In a previous report Bader et al. [1] found that during the first 24 h in culture on collagen-coated dishes the only voltage- and time-dependent current is a potassium current consisting of two components. A voltage-gated sodium current (I_{Na}) appears during the second day in culture. In this paper we have reanalyzed the time of appearance of I_{Na} and of electrical excitability properties in MNC neurons under different coating conditions. Neuronal cells were unambiguously identified by immunocytochemical visualization of neurofilament proteins.

Experimental procedures

Isolation and culture of explants. Japanese quail (*Coturnix coturnix japonica*) eggs were incubated at 38 °C for about 36 h. Migrating mesencephalic neural crest cells were isolated following the procedure described by Ziller et al. [26]. Briefly, crest cells from the superficial ectoderm were excised from embryos at the 9–12 somite stage. Two or three explants at a time were cultured on uncoated or coated plastic dishes (35 mm diameter, NUNC, Roskilde, DK) in serum-free basic Brazeau medium (BBM) [26] without parathyroid hormone. Dishes were coated with rat tail collagen or human plasma fibronectin or EHS mouse tumor laminin. Rat tail collagen (Sigma Chemical Co., St Louis, MO, USA: 0.33 mg/ml in 0.2% acetic acid in distilled water) was applied to plastic dishes and air-dried at room temperature; FN (Sigma or a generous gift of Drs G. Tarone and M. R. Amedeo, obtained according to Tarone et al. [24], 0.1 mg/ml) and LN (Sigma, 0.1 mg/ml) were applied for at least 2 h at room temperature. Before adding the cells the dishes were rinsed twice with phosphate buffered saline (PBS, Gibco Laboratories, Grand Island, NY).

Electrophysiology. Electrophysiological recordings were performed using the whole-cell version of the patch clamp technique. Cell capacitance was measured by analogical compensation. Data were collected by means of an Axopatch-1 C amplifier with a filter cut-off frequency of 2 kHz (Axon Instruments Inc., Foster City, CA, USA), stored on videocassette through a modified

PCM system (Sony) and subsequently digitized at 100-μs intervals and analyzed with an Amiga 2000 (Commodore) computer. Voltage-clamp and specific blocking agents were used to identify the membrane currents. During the experiments, a solution of the following composition was superfused: NaCl (140 mM), KCl (5.4 mM), $CaCl_2$ (3 mM), $MgSO_4$ (0.8 mM), glucose (3 g/l), vitamins (Gibco, 10 ml/l), penicillin (Gibco, 100 U/ml), streptomycin (Gibco, 0.1 mg/ml), HEPES-NaOH (5 mM), pH 7.3. Blocking agents of potassium channels, tetraethylammonium chloride (TEA, 20 mM) and 4-aminopyridine (4 AP, 4 mM), and of sodium channels, tetrodotoxin (TTX, 1 μM), were added to this control solution. Solutions were continuously oxygenated and exchanged in the bath by means of a peristaltic pump. Electrodes had resistances between 5 and 10 Mohm. Two pipette solutions were used: the first, referred to as 'high potassium', contained (in mM) 133 KCl, 3 $MgCl_2$, 5 EGTA, 5 Na_2ATP, 0.4 Na_2GTP, 5 Na_2-phosphocreatine, 5 HEPES-KOH, pH 7.3. In the second intracellular solution ('high cesium'), KCl was isomolarly substituted by CsCl; this solution was buffered to pH 7.3 with HEPES-CsOH. All experiments were performed at room temperature (22–24 °C). The drugs used in the experiments were purchased from Sigma unless specified.

Immunocytochemistry. Investigations were performed using the indirect immunofluorescence technique. Cultures were fixed with methanol at – 20 °C for 3 min and rinsed twice in PBS. Neuron-like cells were visualized using a monoclonal antibody against the 150 K subunit of the neurofilament (NF) proteins (D. Dahl, Boston). Cultures were first incubated with normal goat serum diluted 1:100 in 0.1% Triton-X-100 in PBS, and then with the primary antibody (dilution 1:40), in the same buffer, overnight at room temperature. After being rinsed in PBS, the secondary antibody, goat anti-mouse Ig conjugated with fluorescein isothiocyanate (Janssen Biochimica) diluted 1:20 with 0.1% Triton-X-100 in PBS, was applied for 1 h at room temperature. Cultures were washed in PBS and mounted in a solution containing glycerol and PBS (9:1) with sodium azide to a final concentration of 25 g/l. Cultures were examined and photographed on a Leitz microscope. Appropriate controls, omitting the primary antibody, were performed on parallel cultures.

Statistical analysis. The data were expressed as the mean ± standard deviation. The experimental groups were compared using the chi-square test with Yate's correction or the one way analysis of variance (ANOVA). If a significant F resulted from the analysis of variance ($p < 0.05$), the Student-Newman-Keul's multiple range test was applied to determine where differences were located among the groups.

Results

Identification of neuronal cells. After approximately 18 h in culture and independently of the substrate used, most of the cells in the MNC explants and around them were of polygonal fibroblast-like shape. Around each explant 10–30 cells had bipolar or triangular bodies with long processes and an eccentric nucleus, distinctive of neuronal morphology. Neurite length was 5–10 times the body diameter. In the immunocytochemical experiments, all the cells showing this morphology could be labeled by an antibody against 150 kDa neurofilament protein (fig. 1). We did not observe any marked difference in the number or in the morphology of neurons cultured on different substrates.

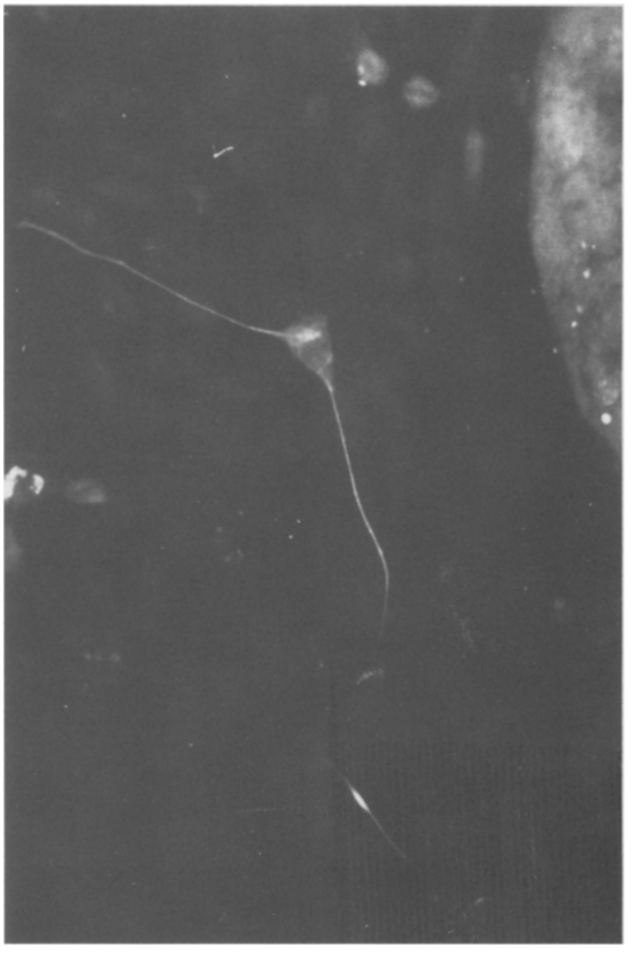

Figure 1. Positive staining with anti-150 kDa neurofilament antibody observed in a cell with neuronal morphology cultured for 24 h on FN substrate. × 328.

Electrical membrane properties of neuronal cells. All electrophysiological experiments were performed on cells cultured for 18–25 h and showing neuronal morphology. A first set of experiments was performed with high potassium in the internal solution. In 54 cells, the resting membrane potential V_m was – 42.0 ± 11.0 mV. After 18 h of culture, all the cells showed well developed outward currents in response to depolarizing voltage steps from a holding potential of – 100 mV, when studied in the voltage-clamp mode (fig. 2A). In some experiments 20 mM TEA and 4 mM 4AP were added to the bath in

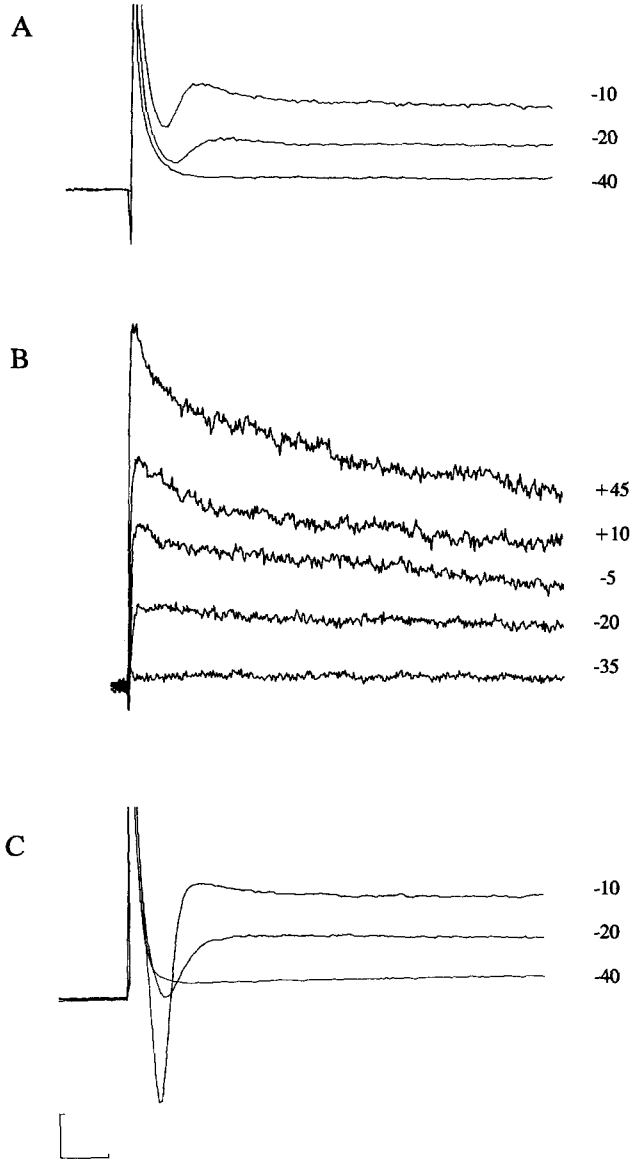

Figure 2. Currents recorded from MNC cells showing neuron-like morphology. *A* Responses to three voltage steps from − 100 mV to the voltages indicated on the right, in a cell that did not show a transient inward component. *B* Difference between the outward currents reported in *A* recorded before and after application of a solution containing 20 mM TEA and 4 mM 4AP. *C* Responses to the same voltage steps as in *A* in a cell that produced an inward current. Cells cultured on collagen (A) and fibronectin (C), culture times 22 h. Calibration: vertical bar 400 pA in *A* and *C*, 150 pA in *B*; horizontal bar 2 ms in *A* and *C*, 30 ms in *B*.

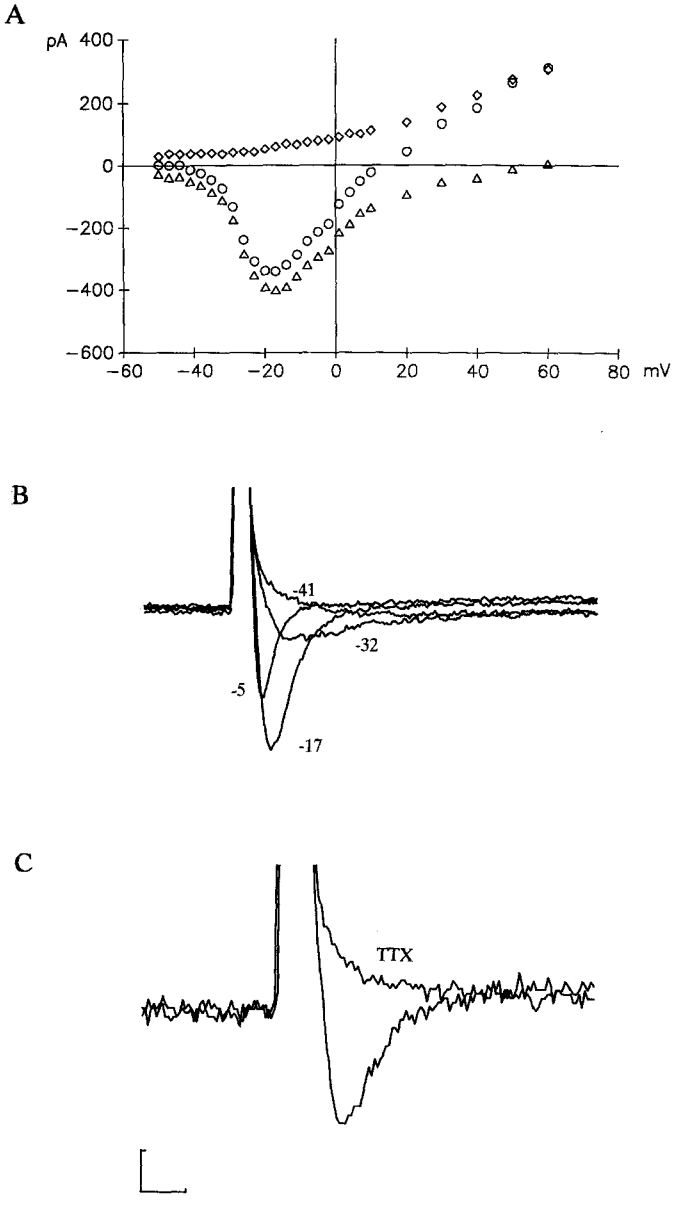

Figure 3. Currents recorded from a cell internally perfused with high cesium solution. *A* I/V relationships obtained from the same cell as in *B*. Circles: current values at the peak of the inward current; diamonds: current values at the end of the 200 ms steps; triangles: difference between circles and diamonds. *B* Responses to four voltage steps from − 100 mV to the voltages indicated. Cell grown on uncoated plastic dish; culture time 20 h. External solution was control solution (see 'Experimental procedures'). *C* Block of the inward transient current by TTX. Current values recorded in response to a voltage step from − 100 to − 14 mV before and after the application of 1 μM TTX. Cell grown on laminin; culture time 20 h. External solution was control solution (see 'Experimental procedures'). Calibration: vertical bar 100 pA in *B*, 50 pA in *C*; horizontal bar 2 ms.

order to block the transient outward and delayed rectifier K^+ currents[2]. Figure 2 B shows the difference between the outward currents recorded before and after application of the two blockers in the same cell of figure 2 A. The maximum amplitude of the total potassium current sensitive to TEA and 4 AP was of the same order of magnitude as those reported previously by Bader et al.[2]. The mean current for 6 experiments (4 FN and 2 COLL) using the above protocol was 1138.2 ± 164.0 pA for a step from − 100 to + 45 mV.

As shown in figure 2 C, in the majority of the cells (32 out of 54) a fast transient inward current could be observed. 126 cells were tested in high $[Cs^+]_i$ in order to abolish, or at least greatly reduce, the outward K^+ currents. Cell capacitance was 9.1 ± 2.9 pF without any significant difference between the four plating conditions. Of these cells, 88 (70%) showed the inward current. Figure 3 A

shows the I–V relationship obtained from one such experiment, and figure 3B shows the current tracings at four different voltages from the same experiment as in A. In this experiment, the difference between the current recorded at the peak and that recorded at the end of the step shows a maximum at − 17 mV and decreases to 0 at + 60 mV, in accordance with the properties of the voltage-activated sodium current in embryonic avian nerve cells [1, 4, 9].

In tests with 40 cells showing the inward current, 1 μM TTX was added to the bathing solution. In all cases a complete reversible block of the inward current was observed (fig. 3C). In 5 cells the reversal potential V_{Na} of the TTX-sensitive current was 49.9 ± 4.9 mV. This value is in good agreement with the theoretical E_{Na} calculated with the internal and external sodium concentrations used in these experiments: 50.0 mV (T = 23 °C).

Role of fibronectin, collagen I and laminin in the development of the voltage-activated sodium current. In order to determine if the different substrates had any role in the occurrence of I_{Na}, the data were divided into four subsets corresponding to the four plating conditions. Figure 4A shows the percentage of cells showing the I_{Na} in the four plating conditions for culture times between 18 and 25 h. Mean culture time was 22 ± 2 h for all the four conditions. The values were: 82.8% for FN-plated cells (n = 29); 84.4% for collagen-plated cells (n = 32); 61.8% for LN-plated cells (n = 34); 51.6% for cells plated on plastic dishes (n = 31). FN and COLL were significantly different when compared with plastic (chi-square test with Yate's correction: $p < 0.05$).

We subsequently considered the mean peak current densities from the four subsets. In each of the four groups, only those cells in which the time to peak for the maximum Na^+ current was less than 3 ms, and in which the voltage of the maximum peak current was < 0 mV, were considered. This was done in order to avoid artifacts in the estimate of the current density due to poor clamping conditions. For the 54 selected cells, t_{peak} was 2.1 ± 0.5 ms and V_{peak} was − 9.7 ± 5.5 mV, without any significant difference between the 4 subsets. Figure 4B shows the results obtained with this procedure: the current density for the FN group was 49.6 ± 41.8 pA/pF (n = 10 from 7 dishes); for the COLL group: 20.2 ± 10.8 (n = 12 from 7 dishes); for the LN group: 18.4 ± 70.0 pA/pF (n = 20 from 9 dishes); for cells on plastic 16.9 ± 14.6 pA/pF (n = 12 from 6 dishes). In this case the analysis of variance gave a highly significant $F = 5.32$ ($p < 0.001$). The FN group was significantly different from the others with a confidence level of 99% (Student-Newman-Keul's test). The high value for the Na^+ current density in cells grown on FN could be compatible with the ability of these cells to generate action potentials: in fact, the I_{Na} density supported the generation of action potential in some cells (fig. 5).

It should be noted that the beginning of an inward current component is not well separated from the capacity spike (fig. 3) which would preclude a kinetic analysis of I_{Na} in terms of activation/inactivation time constants.

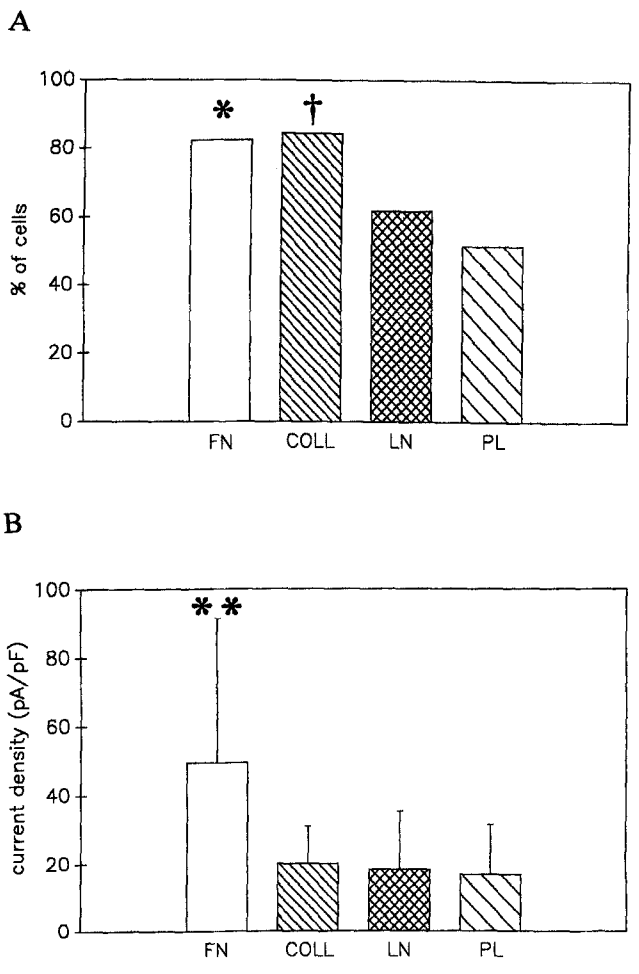

Figure 4. *A* Percentage of cells showing I_{Na} in the 4 plating conditions. Statistically significant difference was found between FN and PL (chi-square test; * $p < 0.05$) or COLL and PL († $p < 0.05$). *B* Mean peak current densities in the 4 plating conditions. Neurons cultured on FN differ significantly (ANOVA followed by Student-Newman-Keul's test; ** $p < 0.001$) from the other three groups. Standard deviations are given as bars.

Discussion

The data presented above show that, in cultured cells from the quail neural crest with a neuron-like morphology and positively stained by anti-NF antibody, a sodium current blocked by TTX can be observed between 18 and 25 h after plating. These are the earliest times at which it is possible to identify neuron-like cells unambiguously. This current was observed in 50–80% of cells, depending on the different substrates used. In some neurons the current density was great enough to allow the generation of action potentials. All neurons tested in this time span had well developed potassium currents. No inward current through Ca^{2+} channels could be observed; this was confirmed by the fact that, after TTX application, no residual inward current could be found.

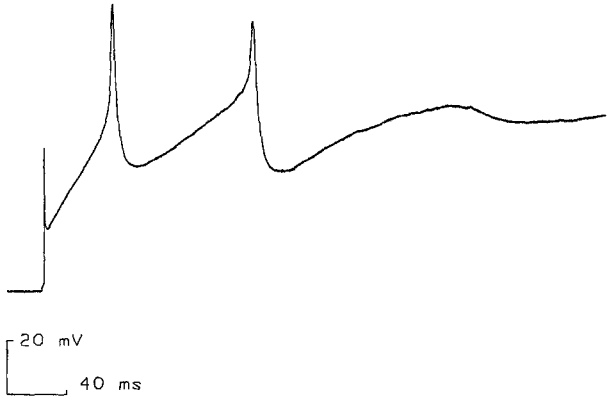

Figure 5. Action potentials recorded from a cell cultured for 25 h on FN. The membrane potential was released from − 100 mV and attained its resting value, about − 40 mV, after generating two spikes.

These data are in agreement with the description of the order of appearance of membrane currents in MNC cells reported before[1]: however, in our findings the appearance of the I_{Na} is shifted to much earlier times (18 h compared with 36 h). It must be noted that the morphology of neurons in our experiments was different from neuronal cell morphology observed by Bader et al.[2] (compare fig. 1 in this paper with table 1 A in Bader et al.[2]); cell morphology in our experiments appears to reflect a more advanced developmental stage and is very similar to that described by Ziller et al.[26] at the same culture times. It cannot be excluded that these discrepancies may be due to differences in cell culture conditions. Bader et al.[2] used collagen-coated plastic coverslips, while we used coated plastic dishes (see 'Experimental procedures'). Substrate adsorption could be influenced by the kind of plastic used.

Different substrates have been utilized in order to test their role in the appearance of the voltage-dependent sodium current and of the electrical excitability in MNC cells. We have observed that cultures grown on FN- and collagen-coated dishes contained a significantly higher percentage of neuron-like cells that show I_{Na} as compared to cells grown on uncoated plastic dishes. Moreover, in cells grown on FN-coated dishes that showed I_{Na}, the density of this current was significantly higher than in corresponding cells from the other three experimental groups.

Our experiments have shown that FN and collagen favour the expression of I_{Na}. In this respect, it is interesting that both molecules are secreted by crest cells in vitro[10,15]; moreover, Greenberg et al.[11] have shown that crest cells are able to adhere to collagen only in the presence of FN. In our experimental conditions some FN appears to be secreted by fibroblast-like cells and can be identified immunocytochemically in the ECM[25]. The addition of a saturating amount of collagen or fibronectin molecules may improve cell adhesion and survival. Our results can thus be explained by a general, trophic effect of these ECM molecules. The additional observation that, in neurons plated on FN, the current density is greater than on other substrates might imply a more specific role of FN in the regulation of the expression of the voltage-dependent inward Na^+ current in neuronal cells derived from the MNC. The idea that FN has an important role in neuronal differentiation in MNC cells is also supported by the observation of Tolosano et al.[25], who found a striking reduction in the number of neurons when MNC explants were grown in the presence of an anti-FN antibody.

No significant differences can be found between cells grown on LN-coated dishes and on plain plastic dishes, either in the percentage of cells showing I_{Na} or in the current density. In this respect it is worth mentioning that, unlike COLL and FN, LN is neither present along the migration pathway[7,18] nor is it secreted in vitro by the crest cells[14]. All three molecules tested are reported to stimulate neurite outgrowth in vitro, LN being the more potent neurite-promoting factor[21]. Differences in neurite number and length could influence the quality of voltage clamping and consequently impair the comparison of current amplitudes in the subsets of cells plated with the different ECM molecules. In our case no morphological differences could be detected qualitatively, but it cannot be excluded that they may be detected by a quantitative analysis. This possibility should not invalidate our data, since we have compared current densities only in cells in which a good voltage clamp was obtained (see 'Results').

In conclusion, the data presented in this paper show that cultured developing neurons from quail MNC consistently exhibit a voltage-activated sodium current and electrical excitability properties at earlier times than reported before[1]. Moreover, our findings provide evidence for a role of two ECM molecules, collagen I and fibronectin, in the neuronal development of MNC cells.

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Serially arranged myofibers: An unappreciated variant in muscle architecture

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Abstract. Our comparative studies suggest that the length of myofibers in tetrapods is subject to an unappreciated degree of variability. Many mammalian strap muscles are composed of short, overlapping myofibers. This arrangement and its associated distribution pattern of motor endplates (neural control) appear to be general in birds and widespread in other tetrapods. Contrariwise, most muscles of primates appear to be composed of long myofibers. The implications of this variation for studies of development, neuromuscular control, and muscle function are largely unexplored.
Key words. Birds; motor endplate; muscle: muscle architecture; pectoralis; myofiber.

Recent studies of muscle architecture have produced data concerning myofiber length that in some ways appear contradictory. The data are further confused by conflicting statements in the literature. Textbooks [1-3] often directly state or strongly imply that the myofibers of parallel-fibered strap muscles in vertebrates run the full length of the muscle. However, there is an extensive physiological, neurological, and anatomical literature, dating back more than a century, that indicates that the myofibers of some mammalian strap muscles do not traverse the muscle, or even its fascicles. Rather, the myofibers are short, serially arranged fibers that overlap broadly. The intrafascicular ends of these fibers are tapered for about 30% of the fiber's length [4-12]. The axons of motor neurons innervating these myofibers branch to cells in each level or tier of the series. Motor endplates (meps) occur in zones or bands oriented perpendicularly to the columns of cells. These bands are revealed by staining for the acetylcholinesterase present in the endplates.

Although the existence of serially arranged muscles containing short fibers is well documented, many investigators appear to be unaware of the phenomenon. Hence, from time-to-time it is 'rediscovered'. A recent revival [9] spurred a series of investigations that together have shown that: 1) Serially arranged fibers are generally missing in species of mammals in which strap muscles are 3 cm or less. 2) Myofibers in strap muscles of the hind leg of many medium-sized (cat [9,13], goat [11,13], pig [14]) to large (cattle [14], giraffe and hippopotamus, pers. comm.) mammals are serially arranged, as are those of the thigh muscles of chickens [15]. 3) The number and relative spacing of mep bands in any given muscle is established at birth [11,14-16]. 4) There are no specialized junctions between myofibers, but cells are bound together by a complex weave of collagen fibers. When a cell contracts, the tension generated is dispersed to and through both the collagen fibers and adjacent, non-contracting cells. The transfer of tension from contracting to non-contracting cells is aided by complex changes in the shape of the contracting cell. These changes help maintain a constant surface area [17]. Therefore, misunderstandings may result if a muscle is thought of as a scaled up sarcomere or as a series of sarcomeres with direct, linear transmission of tension along the series.

Most vertebrate twitch fibers are singly innervated [8,18]. If all cells extend the length of the muscle, and if all are innervated centrally, then one would expect to see a single band of meps more-or-less at the midlength of the muscle. Such a pattern is common in the strap muscles of many small mammals and in primates of all sizes. The presence of many mep bands along the length of a muscle might be explained by either of two possibilities. Long cells might be innervated at different points along their lengths. Alternatively, the muscle might be comprised of