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## Expression of Membrane Currents in Rat Diencephalic Neurons in Serum-Free Culture\*

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The whole-cell gigaseal voltage clamp technique has been used to investigate the timing of expression and type of voltage-dependent ionic currents in dissociated primary cultures of fetal rat (E17) diencephalic neurons grown in a serum-free defined medium. The expression of membrane currents varied among cells at any particular time in culture. Despite this variability, certain characteristics of the appearance of ionic currents emerge from this study. These are: (i) the earliest appearing membrane current is a voltage-dependent outward current carried by  $K^+$ . In some cells, it is the classical delayed rectifier current, whereas in others it is the transient outward current ( $I_A$ ). (ii) The earliest appearing inward current is carried by  $Na^+$ . In some cells the channels are first expressed in the neurites and then in or near the cell body. The early neuritic  $Na^+$  channels are blocked by cobalt or cadmium as well as by tetrodotoxin (TTX). In others, the early  $Na^+$  channels appear in or near the cell body and are only blocked by TTX. (iii) With additional time in culture, a majority of cells exhibit a  $Ca^{2+}$  current at the time of  $Na^+$  channel appearance in or near the cell body as well as a transient  $Ca^{2+}$ -dependent outward current. The  $Ca^{2+}$  current is only a small fraction of the total inward current. These inward currents show the classical pharmacologic profile. The complex pattern of expression of ionic current may reflect multiple populations of neurons with different developmental sequences resulting from differences in cell age and lineage.

### INTRODUCTION

An important component of the overall process of neuronal cytodifferentiation is the expression of membrane channels responsible for active ionic currents. Although previous studies on developing neurons from various animal sources have not demonstrated a consistent pattern of expression of these ionic channels, a number of common features have been described. These include the expression of outward current before the appearance of the inward current<sup>5,6,36</sup> and a shift in the ionic mechanism of the action potential from  $Ca^{2+}$ -dependent to  $Na^+$ -dependent, seen in some but not all neurons<sup>2,6,16,29,34,37</sup>. These modes of expression of inward membrane current have been observed in both invertebrate and vertebrate neurons. Differences in the sequence of expression of ionic channels in neurites and soma have been observed in neurons from vertebrate and invertebrate species<sup>16,23,24,37</sup>. As a result, it is still not

clear whether the expression of ionic channels underlying the action potential undergoes a similar developmental change in all neurons. In particular, very little information is available about the expression of electrical excitability in developing neurons from mammalian brain.

The ability to culture dissociated brain cells in fully defined medium has stimulated new approaches to the study of CNS development under controlled environmental conditions<sup>11,13</sup>. In a variety of culture systems<sup>7,13</sup> dissociated cells from brain and spinal cord rapidly develop the complex morphology displayed by neurons *in vivo* and express characteristic neuronal properties that include evoked and spontaneous action potentials, synaptic potentials, neurotransmitter synthesis and neurotransmitter sensitivity. Primary cultures have proved to be particularly useful for studying neurite development<sup>22,38</sup>, electrical excitability<sup>27,29</sup> and neurotransmitter expression<sup>28</sup>.

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In this paper we describe the characteristics of expression of voltage-dependent membrane ionic currents in dissociated rat diencephalic neurons cultured in a serum-free defined medium. Due to their small size and fragility, developing neurons are easily damaged by microelectrode impalement, limiting reliable recordings to a few seconds to minutes<sup>34</sup>. Consequently we have adapted the tight seal whole cell recording technique<sup>18</sup> to obtain stable recordings for 20 min or more.

## MATERIALS AND METHODS

Brains were obtained from 17-day-old rat embryos under sterile conditions. Following the removal of meninges and blood vessels, the cerebrum was discarded and the diencephalon was dissected free by a transverse cut rostral to the tectum. Tissue pooled from 8–10 fetuses was dissociated enzymatically and cells were cultured in serum-free defined medium according to the procedure described previously<sup>2</sup>, with the following modifications: streptomycin, penicillin A, fungizone and HEPES were omitted from the medium and KCl was reduced from 25 to 5.4 mM. Cells were grown in 24-well plastic dishes on 12-mm glass coverslips (no. 1) precoated with poly-D-lysine.

At the time of plating, cells were completely dissociated and evenly distributed throughout the culture dish. Most (>95%) cells were spherical, 10–15  $\mu\text{m}$  in diameter and devoid of processes although a few cells had rudimentary neurites. These began to develop within 1 h after plating, and by 3 h a majority of the cells had processes extending up to several times the cell diameter. By 24 h in culture, about 85% of the cells were rounded and exhibited neurites, the majority of which were long and highly branched at a distance from the perikaryon. These were considered to be neurons based on characteristic morphology<sup>25</sup> and the ability to bind tetanus toxin<sup>2</sup>. The remaining non-neuronal cells were identified as astroglia, oligodendroglia or fibroblasts on the basis of morphology and immunocytochemical staining<sup>2,30</sup>. These morphological features of the rounded and flat cells were retained throughout the culture period. Since unambiguous discrimination of neurons from astroglia was difficult during electrophysiological experiments, recordings were made from randomly selected rounded cells with one or more long processes.

For electrophysiological recording, a coverslip containing ca. 800 cells/ $\text{mm}^2$  was removed from the culture dish and placed in a plexiglass chamber on the fixed stage of a Zeiss inverted microscope equipped with phase-contrast optics. The chamber was continuously superfused at 0.1 ml/min to a depth of ca. 2 mm with a solution containing 145 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$  and 10 mM HEPES, pH 7.4 (control saline). The chamber temperature was maintained at 37 °C by a local heating circuit. All experimental solutions were filtered with 0.2- $\mu\text{m}$  filters and cells were rinsed 3–5 $\times$  with the filtered solution.

Patch pipettes were fabricated from WPI 150-6 capillary glass and filled with 140 mM potassium, 2 mM  $\text{MgCl}_2$  and 10 mM HEPES pH 7.2. In some experiments 100  $\mu\text{M}$  EGTA was added to the pipette solution to reduce contaminant  $\text{Ca}^{2+}$ . The pipette was placed on the head-stage of a List L/M EPC-5 (1 G $\Omega$  or 10 G $\Omega$  feedback resistor) patch clamp unit and was positioned with a Narashigi hydraulic drive micromanipulator. Pipettes with resistance values of 3–7 M $\Omega$  in control saline were used. Following the establishment of a gigaseal, suction pulses were applied to the pipette interior to establish the tight seal whole-cell recording mode. Membrane currents at later developmental stages (3 days on) were sufficiently large to introduce series of resistance-based artifacts in the current records<sup>18</sup>. Because we were also faced with additional space clamp problems due to cell geometry, we did not regard electronic compensation of series resistance worthwhile and instead we tried to avoid extensive quantitative interpretation of the whole-cell current data. Data were recorded on a Vetter FM-tape recorder and photographed from an oscilloscope. The capacitive transients have been removed from the figures for the purposes of clarity.

## RESULTS

Electrophysiological properties of neurons were examined after 1 h–11 days in culture. The resting membrane potential was measured from the pipette potential under current clamp at zero pipette current after establishing the tight-seal whole cell recording mode<sup>18</sup>. The cell resting potentials ranged between –45 mV and –70 mV with no systematic change from

1 h–11 days in culture. Membrane currents, recorded after applying brief depolarizing voltage steps, superposed upon a holding potential set at  $-40$  mV,  $-60$  mV or  $-80$  mV. Recorded currents remained stable over the duration of an experiment, typically about 10 min.

From 1 to 9 h in culture ( $n = 17$ ) no cells were found to have voltage-dependent currents other than the leakage current. There was no change in the observed currents when the holding potential was hyperpolarized to  $-80$  mV or depolarized to  $-40$  mV, suggesting that the absence of any voltage-dependent current was not due to the inactivation of channels. It is likely that the absence of voltage-dependent currents in the early periods of culture is due to neurons that have not yet expressed membrane currents or to non-neuronal cells which are difficult to distinguish morphologically from neurons at early times in culture.

By 10–54 h in culture ( $n = 63$ ) all but 14% of cells had developed voltage-dependent currents. In 24% of cells examined at this time (designated Type I), application of depolarizing voltage pulses activated only voltage-dependent outward currents. A typical series of membrane currents recorded from a 24-h-old neuron is shown in Fig. 1A (i). Bath application of 20 mM tetraethylammonium (TEA), which blocks voltage-dependent  $K^+$  channels in a wide variety of excitable cells<sup>35</sup>, blocked the outward current (Fig. 1Aii). Under this condition of suppressed outward current, application of depolarizing voltage steps did not activate an inward current, suggesting that inward channels are not present even in the neurites of these neurons. Addition of 1 mM cobalt or 1 mM cadmium did not affect the outward current, indicating that it is not dependent on  $Ca^{2+}$  influx. Fig. 1B shows the current–voltage relationship of this neuron in control saline and in saline containing 20 mM TEA. The outward current is activated at ca.  $-35$  mV and exhibits the voltage-dependent increase in magnitude characteristic of the delayed rectifier found in nearly all neurons<sup>19</sup>.

Another fraction containing 62% of cells in 10–54-h-old cultures (designated Type II) was characterized by the presence of a transient outward current and an all-or-none inward current. These cells exhibited leakage current for depolarizing voltage steps up to  $+35$  mV from a holding potential of  $-60$  mV

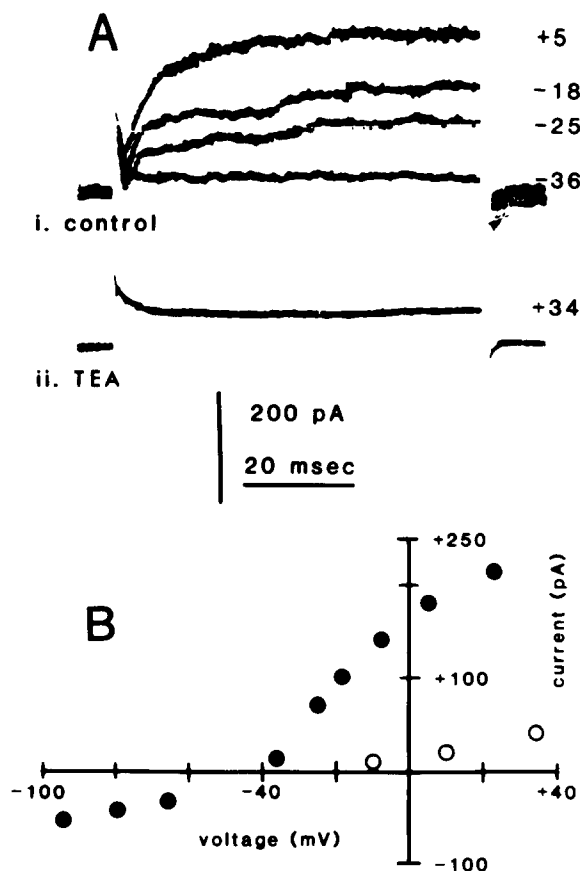


Fig. 1. A: records of outward currents during depolarizations from a holding potential of  $-60$  mV. Transmembrane potential is shown to the right of the current trace; (i) in control saline, (ii) in saline containing 20 mM TEA. No EGTA in electrode; 24-h-old culture. B: current–voltage relationship of the same neuron in control saline (●) and in saline containing 20 mM TEA (○). Currents are typical of Type I cells in 10–54-h-old cultures.

(Fig. 2A); the magnitude of these currents changed linearly with voltage (Fig. 2C). The presence of external TEA (20 mM) or cobalt (1 mM) had no effect on these currents. Application of depolarizing voltage steps from a holding potential of  $-80$  mV instead of  $-60$  mV evoked a transient outward current (Fig. 2B). The amplitude of the transient current was increased by stepping to more positive command potentials. At holding potentials equal to or more positive than  $-60$  mV, the transient outward current was completely inactivated (Fig. 2A). The addition of 20 mM TEA did not significantly affect this current. These characteristics of the transient outward current are similar to those of the  $I_A$  current of molluscan neurons<sup>10,17</sup>. A similar transient  $K^+$  current has

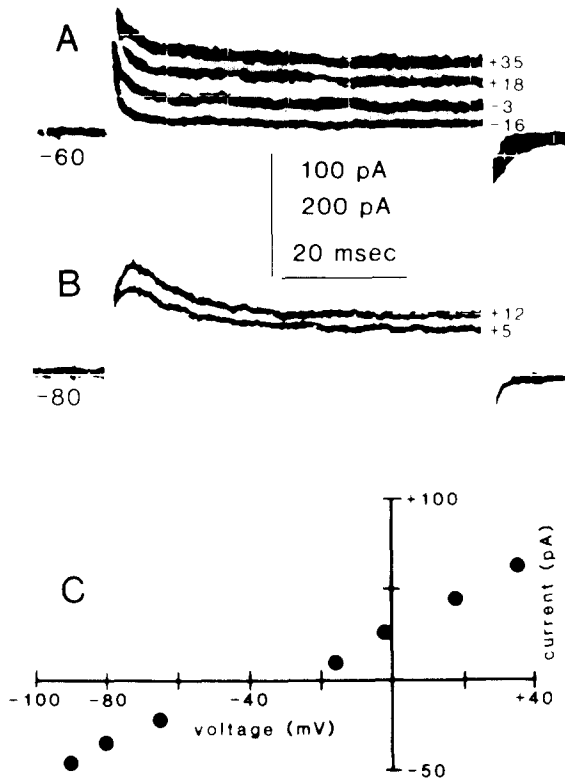


Fig. 2. A: membrane currents of a Type II cell during depolarizations from a holding potential of  $-60$  mV in control saline; 40-h-old culture. B: records of transient outward currents from a 36-h-old cultured neuron. Depolarizing steps were applied from a holding potential of  $-80$  mV. C: current-voltage relationship of the neuron in A. Currents measured near the termination of the voltage pulse.

been observed in cultured rat hippocampal and mouse spinal neurons<sup>33</sup>.

In addition to the transient outward current, this type of neuron (Type II) exhibited a delayed all-or-none inward current for larger depolarizing voltage steps, to  $+40$  mV and above (see Fig. 3). Increasing the voltage step size reduced the latency between the onset of the voltage step and the onset of all-or-none current and increased the frequency of the current spikes. In most cases multiple all-or-none currents were observed (Fig. 3A). The amplitude of the currents was only slightly reduced when the depolarizing step was increased by 40 mV. Since the all-or-none currents persist even when the voltage steps at the soma were above  $E_{Na}$ , it is most likely that the all-or-none currents arose at a remote site where membrane voltage was not clamped. These currents are similar to the currents observed in the neurites of neuroblastoma cells under two-microelectrode voltage clamp<sup>26</sup> and are well described in molluscan neurons<sup>9,20</sup>. The all-or-none currents were not altered in  $Ca^{2+}$ -free saline with 10 mM  $MgCl_2$ , a condition known to block chemical synapses<sup>21</sup>. Also, the currents did not change significantly after 25 min of incubation in  $Ca^{2+}/Mg^{2+}$ -free saline which has been shown to reduce or block direct electrical coupling between cells<sup>32</sup>. Therefore, it is likely that the all-or-none currents were not generated by a synaptic mechanism. However, the all-or-none current could

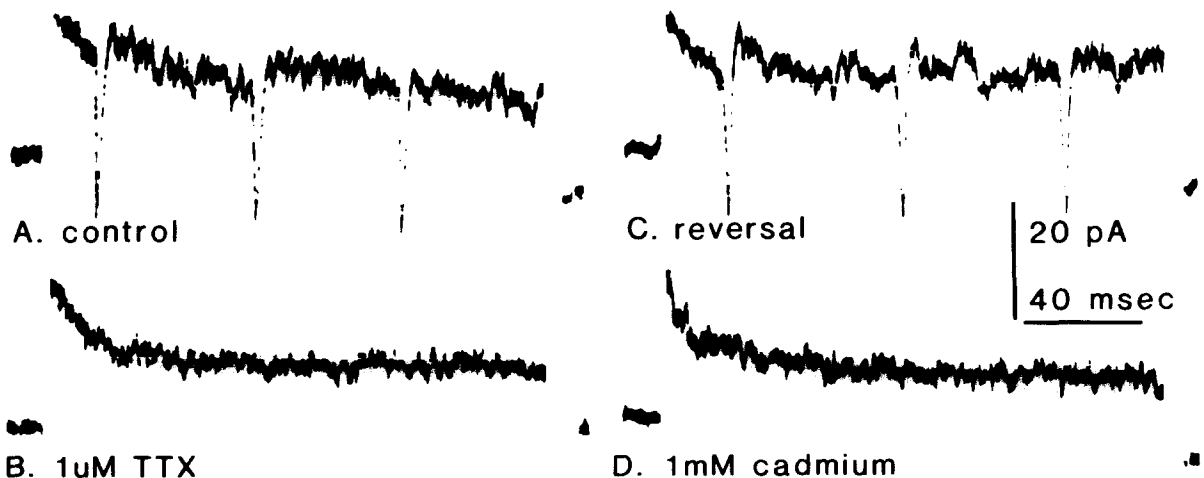


Fig. 3. Multiple all-or-none neurite current recorded from a 36-h-old cultured neuron (Type II). A: in control saline; B: in presence of  $1 \mu M$  TTX; C: reversal from TTX block; D: in presence of 1 mM cadmium. Voltage step to  $+40$  mV from a holding potential of  $-60$  mV.

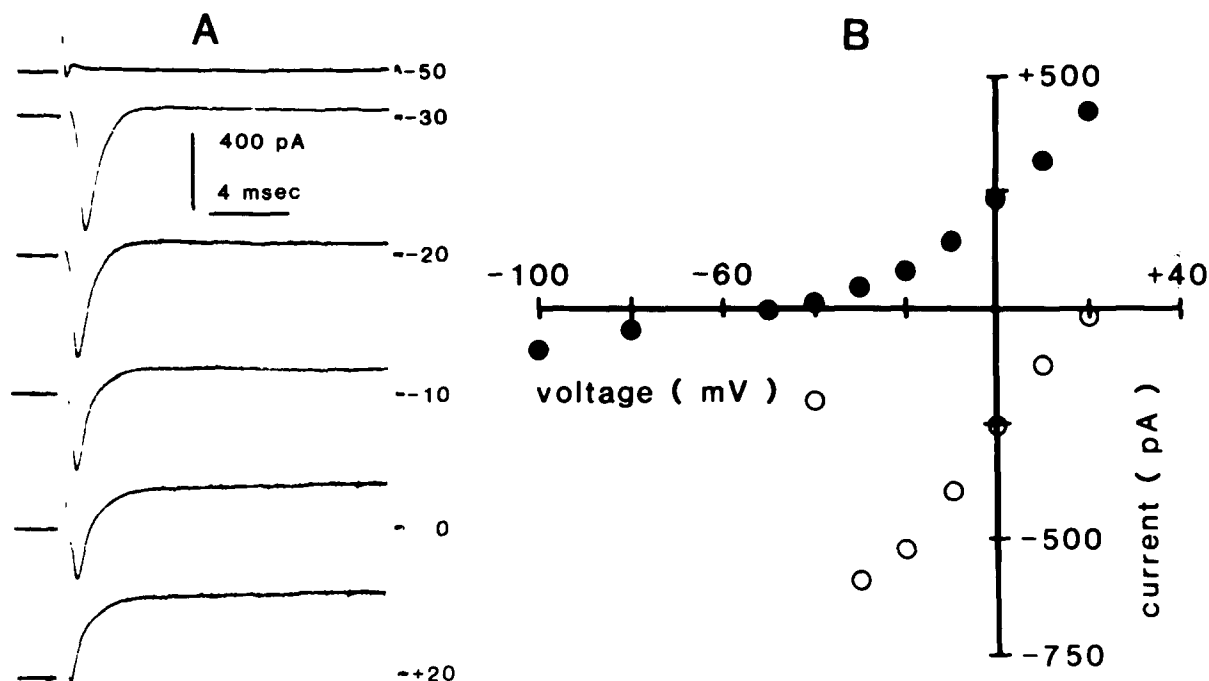


Fig. 4. A: membrane currents recorded from a 6-day-old cultured neuron (Type III) in control saline. Command voltage steps are shown on the right of each current trace. B: current-voltage relationship of the same neuron. Peak inward current (open circles) and outward current (filled circles).

be reversibly blocked by 1  $\mu\text{M}$  tetrodotoxin (TTX; Fig. 3B), or 1 mM cobalt or cadmium (Fig. 3D), in the bathing solution. Replacement of external  $\text{Na}^+$  with tetramethylammonium (TMA) also blocked the all-or-none currents, suggesting that the channels involved are probably  $\text{Na}^+$ -selective. Blockade of Na-channels by extracellular application of cadmium<sup>12</sup> and 1  $\mu\text{M}$  D-600<sup>8,14</sup> have been reported in cardiac cells.

After 68–78 h in culture, cells showed both quantitative and qualitative differences in their membrane currents. Most of these cells had properties of Type II cells. However, the amplitude of the depolarizing voltage step necessary to generate the all-or-none currents was reduced by about 40 mV from that of the 10–54-h-old cells. Also, the amplitude of the all-or-none current increased by a factor of 2. Increasing the step size reduced the latency of the onset of the all-or-none currents and increased the firing frequency. These all-or-none currents also were reversibly blocked by 1  $\mu\text{M}$  TTX, 1 mM cobalt or 1 mM cadmium. The decrease in the threshold and increase in the amplitude of the all-or-none current may be due to changes in the passive properties of the neurite, or to

channel location, behavior and/or density. On the basis of the present study it is not possible to suggest which of these variables contributes to the change in threshold and amplitude of the all-or-none currents.

At this and later times in culture, we found two types (III and IV) of cells with additional membrane currents. Cells of these types dominated the recording samples up to 11 days in culture.

A series of membrane currents recorded from a 6-day-old neuron of Type III is shown in Fig. 4A. Depolarizing voltage steps were applied from a holding potential set at -60 mV. Hyperpolarizing and small depolarizing voltage steps in the range between -100 mV to -40 mV produced small time-invariant leakage currents, whose magnitude changed linearly with voltage. Pulses to levels more positive than -40 mV induced inward current as shown in the left hand records (Fig. 4). The inward current was completely abolished in the presence of 1  $\mu\text{M}$  TTX without affecting the outward or the leakage currents. Essentially the same result was obtained when  $\text{Na}^+$  was replaced with TMA. The presence of 1 mM cobalt or cadmium did not affect the inward current. Outward current began to activate at membrane voltages

around  $-35$  mV. The outward current could be blocked by 20 mM TEA but was unaffected in presence of 1 mM cobalt or cadmium. Fig. 4B shows the current voltage relationship of the peak inward current ( $\circ$ ) and steady-state outward current ( $\bullet$ ) in control saline. The peak inward current reached its maximum value rather precipitously at around  $-30$  mV with the reversal potential at around  $+35$  mV (leakage corrected). The outward current exhibited a voltage-dependent S-shaped rise, seen in other cultured neurons<sup>6,26</sup>. These properties of the outward current of Type III cells were very similar to those of Type I cells.

Fig. 5A shows the membrane currents recorded in control saline from a 5-day-old neuron of Type IV for a command voltage step to  $-15$  mV from a holding potential of  $-60$  mV. A large inward current and transient outward current characterized the neurons of this group. In the presence of external cobalt or cadmium (1 mM), the inward current showed a 17–21% reduction in amplitude, and the duration increased by a factor of 1.7–2.1 (Fig. 5B). The transient outward current was completely abolished. In 1  $\mu$ M TTX, 80–85% of the inward current was blocked but the transient outward current remained (Fig. 5C). Replacement of the external  $\text{Na}^+$  with TMA also blocked 80–85% of the inward current and only slightly affected the transient outward current. These results suggest that roughly 80% of the inward current of cells of Type IV is carried by  $\text{Na}^+$  and the transient outward current is probably a calcium-activated current. The broadening of the inward current in external solution containing 1 mM cobalt or cadmium (Fig. 5B) suggests that in control saline the duration of the inward current is small because there is nearly simultaneous activation of the outward current. These cells (Type IV) showed a voltage-dependent transient outward current ( $I_A$ ) in the presence of 1  $\mu$ M TTX and 1 mM cobalt when the membrane potential was held at  $-80$  mV, similar to those of Type II (Fig. 2).

A series of membrane currents recorded from a Type IV neuron after 7 days in culture is shown in Fig. 6A. Depolarizing voltage steps were applied from a holding potential at  $-60$  mV. The latency between the onset of the voltage pulse and the onset of the inward current, which is quite pronounced for smaller voltage steps, decreases with increasing step

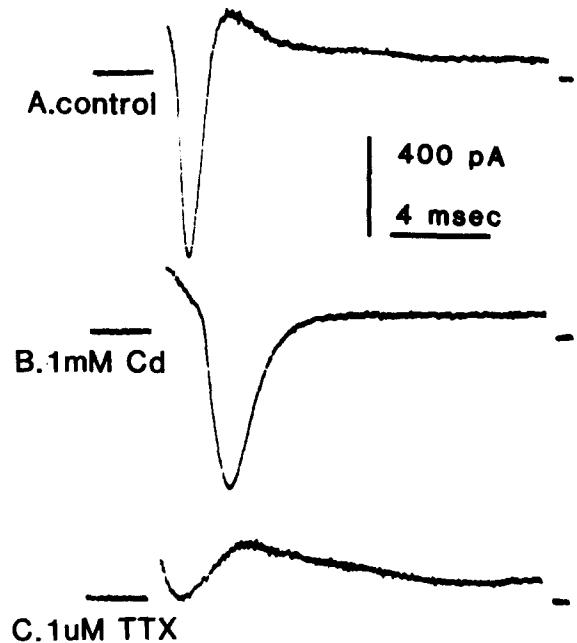


Fig. 5. Membrane currents recorded from a 5-day-old cultured neuron (Type IV). A: in control saline; B: in presence of 1 mM cadmium which eliminated the transient outward current; C: in presence of 1  $\mu$ M TTX, which blocked most of the inward current. Voltage step to  $-15$  mV from a holding potential of  $-60$  mV.

size. This is an indication that at least part of the current is generated in regions of poor spatial control of voltage<sup>9</sup>. Fig. 6B shows, for this neuron, the current–voltage relationship of the peak inward current ( $\square$ ), of the peak value of the transient outward current ( $\circ$ ) and the value of the outward current at the termination of the voltage pulse ( $\bullet$ ). The fast inward current is activated at a membrane voltage slightly above  $-40$  mV, similar to the activation of the outward currents. The reversal potential of the fast inward current is about  $+35$  mV (leakage corrected). The I–V relationship shows that the peak transient outward current reaches a maximum value at  $-10$  mV, where the peak inward current also reaches its maximum. The plateau outward current shows the linear I–V relationship typical of leakage current and indicates that there is little or no delayed rectifier current.

In addition to these groups of cells, two out of a total of 43 cells examined after 9 to 11 days in culture exhibited large voltage-dependent fast and slow inward current, shown in Fig. 7A. Both the fast and the slow components of the inward current showed volt-

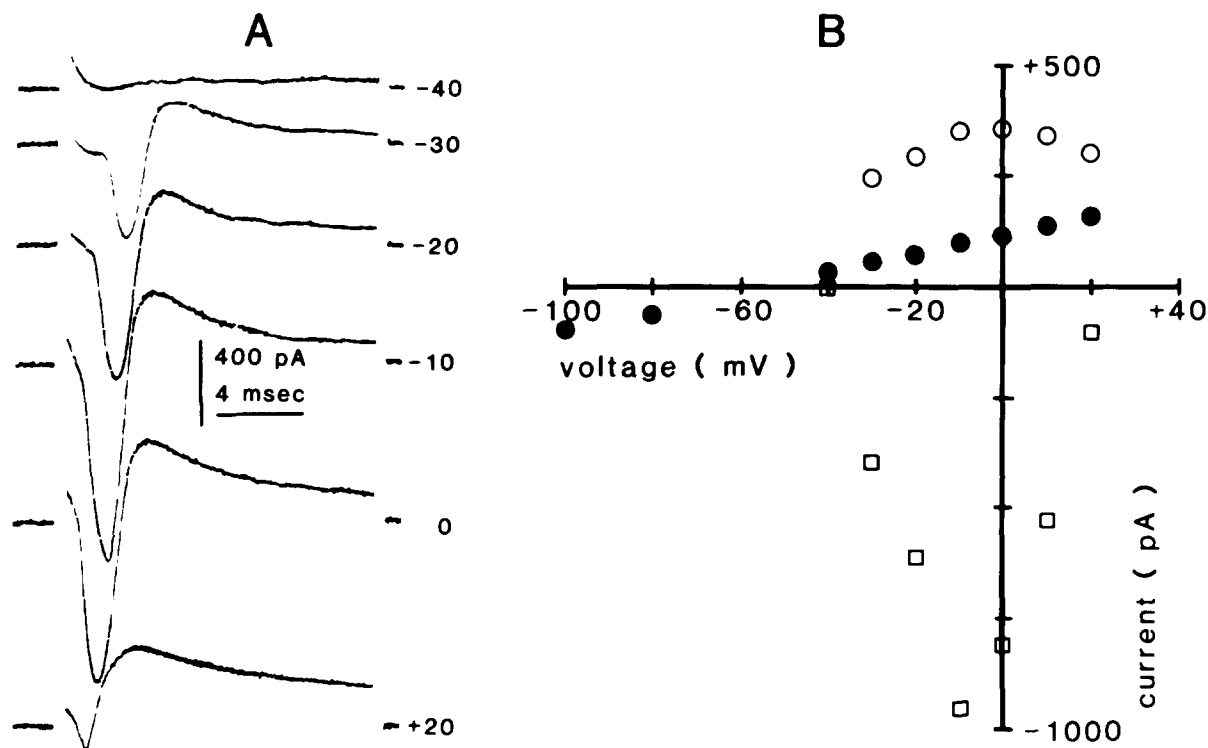


Fig. 6. Membrane currents recorded from a 7-day-old neuron (Type IV) in control saline. Command voltage is shown on the right of each current trace. B: current-voltage relationship of the same neuron. Peak inward current (open squares), peak transient outward current (open circles) and plateau outward current (filled circles).

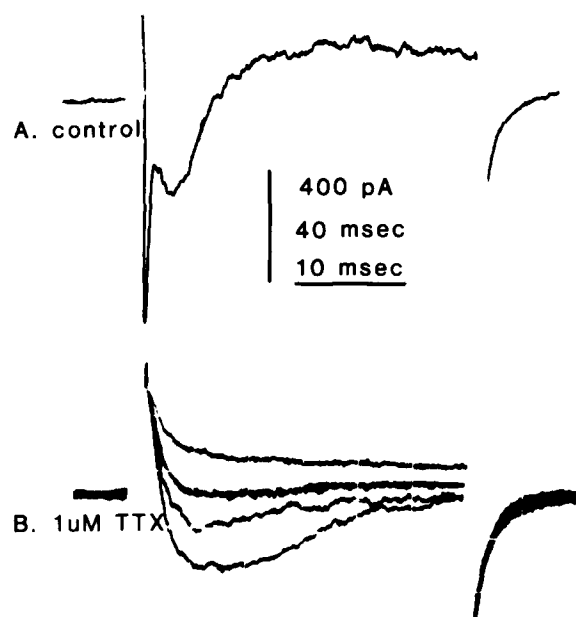


Fig. 7. Membrane currents recorded from a 11-day-old neuron. A: both the fast and slow inward currents at a slower time scale (bar, 40 ms). B: the slow inward current in presence of 1 μM TTX (bar, 10 ms).

age dependency. The fast current was insensitive to 1 mM cobalt or cadmium, but was completely blocked in presence of 1 μM TTX (Fig. 7B). The slow inward current was insensitive to TTX, but was blocked in presence of 1 mM cobalt or cadmium. Because they represent only 1% of the cells studied, they are not included in the tables.

#### DISCUSSION

This investigation was carried out to examine the expression of ionic currents in dissociated rat diencephalic neurons as a function of time in a serum-free culture medium. It is recognized that dissociation itself involves procedures and conditions which clearly affect and may compromise normal neuronal function. Nevertheless, cells which survive this process develop most, if not all, of the functional properties of mature neurons<sup>13</sup> and thus offer unique opportunities for the investigation of cellular mechanisms in a controlled environment.

The freshly dissociated cells are almost completely devoid of processes having the appearance of undifferentiated neurons. It is possible that some of the cells have lost processes during the dissociation procedure. However, it is generally considered that in mammalian CNS, cells which have begun the process of differentiation do not survive the dissociation procedure<sup>11</sup>. In a separate study, we have found that more than 95% of the cells dissociated from 17-day-old fetal rat diencephalon have recently arrested and remain in G<sub>1</sub>-phase of the cell cycle, prior to initiation of differentiation (Ahmed and Fellows, in preparation). Therefore, it is likely that the membrane properties observed in this study are those expressed during the subsequent differentiation of these neurons in culture.

Although the dissociated diencephalic cells are essentially synchronized in terms of the cell cycle, they are unlikely to be of one cell lineage. Since neurons of a number of nuclei of the rat diencephalon that form during the 16th and 17th day of gestation<sup>3,4</sup> would be expected to survive the dissociation procedure, our observation of cells with differing membrane properties may reflect lineage heterogeneity. While it would be desirable to eliminate the problems of lineage heterogeneity by studying the same cell repeatedly, this is not possible because of the small size and fragility of these cells. Consequently, we have investigated populations of cells as a function of time in culture and have characterized cells at each stage according to their observed membrane currents.

Although we have observed variation in the expression of membrane currents among cells at any particular time in culture, certain characteristics of appearance of ionic currents emerge from this study. Firstly, in all cells the outward current appears earliest in development. However, two distinct types of

outward currents were present. Very early in culture, 24% of the cells exhibited the delayed rectifier current and in 62% the transient I<sub>A</sub>-like outward current. Secondly, initially the inward current is carried by Na<sup>+</sup>. In some cells the channels are first expressed in the neurites and then in or near the cell body. The early neuritic Na<sup>+</sup> channels are blocked by Co or Cd as well as TTX. In others, the early Na<sup>+</sup> channels appear in or near the cell body and are only blocked by TTX. In a previous study<sup>2</sup> with 20-day-old fetal cultured brainstem-cerebellum neurons, it was reported that the cells first expressed a Ca<sup>2+</sup>-dependent action potential and then a predominantly Na<sup>+</sup>-dependent action potential. The chronological grouping in that study was much broader which may have led to a premature conclusion, especially given the fact that in a large number of diencephalic neurons cobalt or cadmium also blocks the Na<sup>+</sup> channels. On the other hand, the sequence of expression of ionic currents in 20-day-old brainstem-cerebellum neurons may be different. Thirdly, with additional time in culture, a majority of cells exhibited a Ca<sup>2+</sup> current at the time of appearance of Na<sup>+</sup> channels in or near the cell body as well as a transient Ca<sup>2+</sup>-dependent outward current. These inward currents show the classical pharmacologic profile.

Table I summarizes the 4 different types of membrane currents and Table II the number of cells exhibiting these properties at each time period. Cells displaying Type I properties are present only during the initial period of culture. Their characteristic membrane property is a voltage-dependent, TEA-sensitive outward current. Since the outward current exhibits the I-V relationship typical of adequately voltage-clamped cells<sup>19</sup>, the ionic channels are probably located at or near the cell body. Cells of Type III exhibited a similar outward current along with a TTX-sensitive, cobalt/cadmium-insensitive inward current, graded with applied voltage. The inward current in these cells is carried by Na<sup>+</sup> and the channels responsible for carrying the bulk of the current are probably located in or near the soma. Cells of Type III were only seen after about 3 days in culture. Because of the similar properties of the outward current and the time sequence in which the cells of Type I and III are seen in culture, it is possible that Type I and III properties belong to the same class of neuron, with those of Type III being a more differentiated

TABLE I

*Grouping of cells according to their observed membrane properties*

| <i>Type I</i>       | <i>Type II</i>   | <i>Type III</i>                           | <i>Type IV</i>   |
|---------------------|--|---|--|
| I <sub>K</sub> (V)* | I <sub>A</sub> (V)<br>I <sub>Na</sub><br>(all-or-none) | I <sub>K</sub> (V)<br>I <sub>Na</sub> (V) | I <sub>A</sub> (V)<br>I <sub>K</sub> (Ca), transient<br>I <sub>Na</sub> (V)<br>I <sub>Ca</sub> (V) |

\* Voltage-dependent

TABLE II

Grouping of cells according to their time in culture

Total numbers of cells examined at each time interval as well as the number (percent of interval total) exhibiting properties of Types I–IV.

| Time in culture | Total no. of cells examined | Type I   | Type II  | Type III   | Type IV  |
|-----------------|-----------------------------|----------|----------|------------|----------|
| 10–54 h         | 71                          | 17 (24%) | 44 (62%) | 0          | 0        |
| 68–78 h         | 53                          | 0        | 36 (68%) | 13 (24.5%) | 3 (5.6%) |
| 4–5 days        | 36                          | 0        | 8 (22%)  | 15 (41.7%) | 13 (36%) |
| 6–7 days        | 36                          | 0        | 0        | 14 (38.9%) | 22 (61%) |
| 9–11 days       | 41                          | 0        | 0        | 13 (31.7%) | 28 (68%) |

version of Type I. Studies with cultured avian mesencephalic neurons have shown that the voltage-dependent  $K^+$  currents are expressed first, followed by the expression of  $Na^+$  currents and in some cells  $Ca^{2+}$  currents<sup>6</sup>. A progression from Type I to Type III would be consistent with such a sequence, although alternative mechanisms are not ruled out.

Cells of Type II showed an all-or-none inward current generated at the neurites and a transient outward current similar to the A-current generated in the molluscan cell body<sup>10</sup>. The all-or-none inward current was observed only when the soma was depolarized to +40 mV. Since the all-or-none currents were not affected by blocking both chemical and electrical synapses, it is certain that these currents were generated in the neuron under investigation. Also, since these currents were only slightly affected by voltage steps near or above  $E_{Na}$ , the all-or-none currents must arise at electrically distant neurites where membrane voltage is not under space clamp. The all-or-none currents were abolished by the addition of TTX or by the removal of external  $Na^+$ , indicating that they were carried by  $Na^+$ . However, the blockade of the all-or-none current by cobalt or cadmium raises a question about the nature of the early ionic channel. Cadmium has been shown to block  $Na^+$  channels in cardiac cells<sup>12</sup>. It is also possible that the channels expressed early during development are  $Na^+$  channels having some structural feature common to the  $Ca^{2+}$  channels as suggested for neuroblastoma cells<sup>31</sup> and rat cardiac myocytes<sup>39</sup>. Cells of Type IV also show some of the Type II membrane properties in addition to a large inward current generated in or near the cell body and a  $Ca^{2+}$ -dependent transient outward current. About 85% of the inward soma current is carried by  $Na^+$  and the remainder by

$Ca^{2+}$ , both of which exhibit classical pharmacological specificity. Again, because of the similarities in membrane properties of Type II and IV cells and the time sequence in which the cells of Type II and IV appear in culture, one can speculate that cells of Type IV are a more developed version of Type II cells. The proposed sequence of expression of membrane current would be similar to that of the developing neurons of grasshopper, where  $Na^+$  current is first expressed in the axons and, after further maturation of the neurons, in the cell body<sup>15,16</sup>.

Based on electrophysiological criteria, this study has suggested that, in dissociated primary culture of E17 fetal rat diencephalon, at least two and perhaps more, classes of neurons with different developmental properties are present. In the simplest model, one class of cells first develops an inward current in the neurites and subsequently in the cell body. The neurite inward current is largely carried by  $Na^+$ , but the channel may have structural similarity with the  $Ca^{2+}$  channel in early stages of development. At later stages the inward channels exhibited classical pharmacological specificity. The second class of neurons first expresses a voltage-dependent outward current in the soma and then expresses voltage-dependent inward  $Na^+$  current in the soma. Both the inward and outward currents show classical pharmacological specificity.

This study has demonstrated that the expression of ionic channels in the membrane of the rat diencephalic neurons can take place in the absence of systemic hormones known to influence the development of the brain. Since the serum-free defined medium does not need to contain hormones or trophic factors other than insulin for the survival of these cells in culture<sup>2</sup>, it provides an excellent opportunity for the investiga-

tion of basic mechanisms underlying neuronal differentiation.

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