Early Development of Voltage-Gated Ion Currents and Firing Properties in Neurons of the Mouse Cerebral Cortex

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Abstract

Voltage and current clamp recordings were made from acute slices of mouse cerebral cortex from embryonic day 14 to postnatal day 17. We targeted cells in the migratory population of the embryonic intermediate zone (IZ) and in deep layers of embryonic and postnatal cortical plate (CP). IZ neurons maintain fairly consistent properties through the embryonic period, all expressing high input resistance, inward Na⁺ currents and outward K⁺ currents, and none showing any hyperpolarization-activated currents. In CP neurons, several changes in physiological properties occur in the late embryonic and early postnatal period: inward Na⁺ current density is strongly upregulated while outward K⁺ current density remains almost unchanged, input resistance drops dramatically, and a hyperpolarization-activated current resembling Iₘ appears. As a result of these changes, the action potential becomes larger, shorter in duration, and its threshold shifts to more negative potentials. In addition, CP cells become capable of firing repetitively and an increasing fraction show spontaneous action potentials. This coordinated development of ion channel properties may help to time the occurrence of developmentally relevant spontaneous activity in the immature cortex.
Intro

Spontaneous electrical activity plays a fundamental role in many aspects of nervous system development, including DNA synthesis and cell cycle exit (Dutton et al. 1993; LoTurco et al. 1995; Catalano et al. 1997; Barish et al. 1998; Fields et al. 2001), cell migration (Komuro and Rakic 1992, 1993, 1998; Flint et al. 1999; Behar et al. 2001; Edgar and Price 2001; Maric et al. 2001), formation and refinement of synaptic connections (Shatz and Stryker 1988; O’Leary et al. 1994; Herrmann and Shatz 1995; Shatz and Katz 1996; Catalano and Shatz 1998; Penn et al. 1998), and development of mature ion channel properties (Spitzer 1991; Dallman et al. 1998; Moody 1998; Spitzer and Ribera 1998). The existence and patterns of spontaneous activity in any given neuron early in its development depend on both the input it receives (from any existing synaptic inputs and the presence of non-synaptically released transmitters in the extracellular space; Owens et al. 1996; Haydar et al. 2000), and on its intrinsic ion channel properties.

It has been well-established in invertebrate and non-mammalian vertebrate cells that the properties of ion channels present at early stages of development can be quite different from those in the mature state. These differences are critical in determining the existence of spontaneous electrical activity and in regulating its ability to mediate activity-dependent developmental events (O’Dowd et al. 1988; Wong et al. 1993; Gu and Spitzer 1995; Linsdell and Moody 1995; Greaves et al. 1996; Catalano et al. 1997; Dallman et al. 1998). Because the properties and types of ion channels present in a cell can change rapidly during development, understanding the roles of immature ion channel properties in activity-dependent development has required detailed maps of ion channel development (Greaves et al. 1996; Dallman et al. 1998). Such maps have not been drawn
for developing neurons of the mammalian neocortex in sufficient detail to allow
correlations to be determined between the patterns of ion channel development and the
occurrence of critical developmental periods of spontaneous electrical activity. Here, we
report measurements in neurons of mouse sensorimotor cortex of input resistance ($R_{in}$),
inward Na$^+$ current ($I_{Na}$), outward K$^+$ current ($I_{K}$), and inwardly rectifying currents
between embryonic day 14 (E14) and postnatal day 17 (P17). Our results show that each
of these properties is regulated in a different way during embryonic and early postnatal
development, and suggest that early postnatal periods of spontaneous activity may in part
be regulated by the developmental expression of currents which support repetitive firing
ability in individual neurons.

The neurons that populate the layers of the mammalian neocortex arise in part
from repeated divisions of precursors in the neocortical ventricular zone (VZ), a
proliferative population which in rodents actively generates neuronal precursors during
most of the latter third of embryonic development (Luskin et al. 1988; Takahashi et al.
1994). A secondary proliferative population, responsible for generating most cortical glia,
persists much later in development (Goldman 1995; Levers et al. 2001). During
neurogenesis, which in mouse occurs between about E11.5 and E17, depending on
neocortical region, a steadily increasing fraction of the proliferative population exits the
cell cycle in the VZ and migrates outward through the Intermediate Zone (IZ) to final
destinations in the Cortical Plate (CP) (Sidman and Rakic 1973; Shoukimas and Hinds
1978; Caviness 1982). Presumptive neurons that exit the cell cycle at earlier days occupy
the deeper cortical layers, and those that exit later migrate through these to form the more
superficial layers. This radial migratory process appears to generate the pyramidal cell
population (Mione et al. 1997; Tan et al. 1998; Anderson et al. 1999). More recent
evidence indicates that a large fraction, if not all, of the inhibitory interneurons of the
rodent cortex arise in the VZ of the Ganglionic Eminences, the precursors of the striatum
in the adult animal (Anderson et al. 1999, 2001; Parnavelas et al. 2000).

In various studies of membrane properties of the embryonic rodent neocortex,
proliferating populations have been found to primarily express voltage-gated K\(^+\), Ca\(^{2+}\)-
activated K\(^+\) and small Na\(^+\) conductances when input resistances are sufficient to permit
detection of small currents (Martin-Moutot et al. 1987; Bulan et al. 1994; Mienville et al.
1994; Mienville and Barker 1997; Hallows and Tempel 1998; Noctor et al. 2002; Picken
Bahrey and Moody 2003). Na\(^+\) currents then increase throughout development as cells
begin to migrate and differentiate (Couraud et al. 1986; Huguenard et al. 1988; Hamill et
al. 1991; Mienville et al. 1994; Villegas et al. 1994; Luhmann et al. 2000; Picken Bahrey
and Moody 2003). Changing levels of expression of L-type, LVA, and HVA Ca\(^{2+}\)
currents (Lorenzon and Foehring 1995; Tarasenko et al. 1998), as well as voltage-gated
inactivating (I\(_A\)) and delayed rectifier K\(^+\) currents (Hamill et al. 1991; Mienville and
Barker 1997; Korngreen and Sakmann 2000; Bekkers 2000), occur during both
embryonic and postnatal development. Cells of the developing cerebral cortex gain the
ability to generate activity as early as E18 (Hamill et al. 1991; Luhmann et al. 1999), and
there is some evidence that both Na\(^+\) and outward K\(^+\) currents can be modulated by
activity patterns (Desai et al. 1999). Here we show that presumptive neurons migrating
through the Intermediate Zone have relatively consistent electrophysiological properties
that change very little with developmental stage: high input resistance, delayed K\(^+\)
currents, small inward Na\(^+\) currents, and no hyperpolarization-activated currents. After
they enter the Cortical Plate, however, these properties begin to change. Input resistance drops dramatically by P12. Na\(^{+}\) current density increases while K\(^{+}\) current density remains constant. A slow, hyperpolarization-activated current resembling \(I_h\) appears abruptly at about P6. During the period in the CP, neurons also gain the ability to fire repetitive action potentials during long depolarizing stimuli, and many begin to show spontaneous, repetitive action potentials.

**Materials and Methods**

**Animals**

Timed pregnant C57Bl/6 mice were obtained from ATL, Kent, WA. Pregnant females were euthanized on gestational day 13-18 (E13 – E18) by CO\(_2\) inhalation, according to NIH and institutional guidelines. Both uterine horns were dissected out and placed in ice cold prenatal artificial cerebral spinal fluid (preACSF (mM): 119 NaCl, 2.5 KCl, 1.3 MgCl\(_2\), 2.5 CaCl\(_2\), 1 NaH\(_2\)PO\(_4\), 26.2 NaHCO\(_3\), 11 d-Glucose (Mooney et al. 1996); unless otherwise noted, all chemicals were obtained from Sigma, St. Louis, MO, USA) bubbled with carbogen (95% O\(_2\)/5% CO\(_2\)). Pups were removed from the uterus, staged by visual inspection or crown to rump length, and cerebral cortices were dissected out and placed in ice cold preACSF, keeping intact the section from olfactory lobes through a portion of the brainstem. Some brains were then embedded in a 3% solution of Type IX-A Ultra-low temperature gelling agarose in preACSF that had cooled below 32\(^\circ\)C, and placed on ice until the agarose was gelled. Others were mounted directly to a metal pan for slicing.
Postnatal pups were euthanized according to institutional guidelines, and cerebral cortices were removed to ice cold postnatal ACSF (postACSF (mM): 115 NaCl, 4.3 KCl, 2CaCl₂, 2 MgCl₂, 0.28 MgSO₄, 0.22 KH₂PO₄, 0.85 Na₂HPO₄, 27 NaHCO₃, 25 D-Glucose (Beier and Barish 2000)) bubbled with carbogen. Olfactory lobes and brainstem were removed, and cerebral cortices were mounted directly on a metal pan for slicing.

200 µm coronal slices were cut using a Vibratome 1000 (Technical Products International, St. Louis, MO, USA), removed from the agar if embedded, and allowed to recover in oxygenated room temperature preACSF or postACSF for 60 to 90 minutes before recording.

Voltage Clamp Recordings

Pipets were pulled to a resistance of 8-12 MΩ from 50 µl hematocrit glass capillary tubes using a Narishige two-stage puller (PP-83 and PP-830, Japan), coated with Sylgard 184 (Dow Corning Corporation, Midland, MI, USA), and filled with potassium internal solution (Potassium Methylsulfate (mM): 113 KMSO₄ (ICN Biomedicals), 20 KCl, 10 HEPES, 2, MgATP, 3 Na-ATP, 0.2 Na-GTP, pH to 7.25; or Potassium Gluconate (mM): 100 KGluconate, 0.5 EGTA, 5 MgCl₂, 40 HEPES, 2 Na-ATP, 0.3 Na-GTP, pH to 7.25). In a few cases 3000 MW biotin dextran (2%; Molecular Probes, Eugene, OR, USA) or Neurobiotin (2%; Vector Laboratories, Burlingame, CA, USA) were added to the recording pipet for later confirmation of cell morphology.

Voltage clamp experiments were performed using the whole cell patch clamp technique (Hamill et al. 1981). All recordings were made at room temperature (24-26°C) in preACSF or postACSF. Slices from the somatosensory cortex were chosen for
recording and were placed in a 1.2 ml recording chamber and perfused at a rate of 0.6 ml/min with carbogen bubbled preACSF or postACSF.

Individual cell somas were visualized with an upright Axioskop (Zeiss, Germany) using a water immersion 63x objective with DIC optics (Fig. 1C). Cells within a region up to 200 µm dorsal to the striatal border were targeted prenatally, and within 300 µm dorsal to the caudatopallial angle postnatally. In prenatal recordings IZ cells located in the middle of the lateral IZ, and CP cells located in the middle of that region were targeted (Fig. 1A). In postnatal recordings, cells with a pyramidal morphology were targeted (Fig. 4A).

Pipets were lowered onto individual visualized cells, keeping constant positive pressure to avoid clogging. Pressure was then turned off when the cell was touched, and a seal greater than 4 GΩ usually formed within 2-10 seconds. A holding potential of -60 mV was applied, and brief pulses of suction were applied until the membrane inside the pipet ruptured. Recordings were made using a List EPC7 (Heka Elektronik, Lambrecht/Pfalz, Germany) or Axopatch 1-D (Axon Instruments, Foster City, CA, USA) amplifier. The resulting currents were filtered at 1 kHz, and recorded and analyzed using pCLAMP8 software (Axon Instruments, Foster City, CA, USA).

Most cells were also held under current clamp to investigate activity.

Histology

Slices with cells filled with Neurobiotin or biotin dextran were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.2) for one hour at room temperature or overnight at 4°C. After 4 washes in PBS, the slices were treated with 3% hydrogen peroxide for 10
minutes and rinsed in 0.3% Triton-X 100 in PBS (PBS-TX). Slices were incubated for 2 hours in an avidin-biotin horseradish peroxidase BSA solution (Vector Laboratories ABC kit with 2% bovine serum albumin added) and again washed 4 times in PBS-TX. Fills were developed with diaminobenzidine and glucose oxidase, βglucose, NH₄Cl, NiCl and dehydrated through an ethanol series. Slices were cleared using cedarwood oil or xylene and mounted on slides in DPX (Fluka, Switzerland). Images were acquired with a ZVS3C75DE Digital Camera system (Zeiss) mounted on an Axioskop upright microscope with Optronics software (Optronics, Goleta, CA, USA). Images were stored using Adobe Photoshop 6.0 (Adobe Systems Incorporated, San Jose, CA, USA).

*Analysis methods*

Input resistance ($R_{in}$) was calculated from the average of responses to voltage pulses to ±10 mV and ±20 mV from -60 mV. A triangle wave voltage command was then played to the cell and capacitance measurements were calculated from the amplitude of the resulting square-wave current (Moody and Bosma 1985). Current density was calculated as peak current divided by the capacitance of the cell. Kinetics of activation and inactivation were determined by fitting exponential curves to the appropriate portions of the current trace within PCLAMP8 software. Histograms were created in Sigmaplot (SPSS Science, Chicago, IL, USA), and statistics (descriptive and Student $t$ test) were done in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Figure data are shown as mean ± SEM, with n values noted.
Results

Development of Na\(^+\) and K\(^+\) currents in Intermediate Zone cells

We recorded from 103 IZ cells in animals aged E13 to E18 (see Fig. 1B). Mean input resistance of the IZ cells did not change significantly across embryonic days, ranging from 7350 ± 2594 M\(\Omega\) at E16 (n=17) to 4085 ± 714 M\(\Omega\) at E17 (n=7; p=0.44; Fig. 1D). Capacitance also changed very little during these stages, although the difference between the maximum value of 6 ± 1 pF at E17 and the minimum of 4 ± 0.3 pF at E18 was marginally significant (p=0.046; Fig. 1E). Our previous work showed that in embryonic mouse cortex, dye coupling as seen in Fig. 1B is not associated with significant electrical coupling, so that voltage clamp measurements are accurate for the recorded cell without resorting to uncoupling methods (Picken Bahrey and Moody 2003).

In previous work (Picken Bahrey and Moody 2003), we showed that the amplitude of the inward Na\(^+\) current increased as presumptive neurons exit the cell cycle in the VZ and migrate into the IZ. This implies that I\(_{Na}\) amplitude is determined by developmental stage of individual cells, i.e. by state of migration and time since cell cycle exit, rather than by chronological age of the embryo. If this is true, then mean I\(_{Na}\) amplitude in IZ cells should be similar at different stages, because the IZ is a constantly changing population of cells which began migration at a similar time interval following cell cycle exit.

As shown in Figure 2A (also see insets Fig. 3E), I\(_{Na}\) amplitude was similar in IZ cells at most of these stages, with no significant differences between any pair of stages.
except E13 and E14-16 (E13: 24 ± 7 pA, n=9; E14: 58 ± 6, n=40; p=0.02; data included only from cells with measurable $I_{Na}$). This indicates that presumptive neurons acquire functional $I_{Na}$ as a result of individual differentiation and migration, not as a function of overall developmental time. Because capacitance of IZ cells did not vary during this period (Fig. 1E), $I_{Na}$ density in IZ cells was also constant during this interval of development. There was, however, some variation in the fraction of IZ cells expressing detectable $I_{Na}$, decreasing from a high of 100% at E16 to a low of 67% at E18 (p=0.021 by Fisher’s exact test; Fig. 2B).

The other major current in these cells is a delayed outward $K^+$ current, which is present in virtually all cells at all stages (102/103), as it is in VZ cells (Picken Bahrey and Moody 2003; Albrieux et al. 2002). Unlike $I_{Na}$, the mean amplitude of $I_K$ decreases steadily by about 50% between E13 and E18 (392 ± 62 pA at E13 to 157 ± 46 pA at E18, n=11 and 12, respectively; p=0.008; Fig. 3A, insets in 3E). Because capacitance does not change during this period (see Fig. 1E), average $I_K$ density declines similarly. In addition to the decrease in amplitude, the activation kinetics of $I_K$ slowed somewhat between E13 and E17 (E14: 0.9 ± 0.1 ms, n=41; vs. E17: 5.3 ± 1.4 ms, n=5; p=6.9 x 10^{-10}; Fig. 3C). Neither the voltage dependence nor the inactivation of the outward $K^+$ current change during this time (Fig. 3E).

Closer analysis of $I_K$ amplitudes suggests that the decrease in amplitude may represent a loss of cells with large $I_K$ amplitudes, rather than a shift of the entire population to lower amplitudes. Figures 3B & D compare frequency histograms of $I_K$ amplitudes at E13-15 and E16-18. At the earlier stages, the distribution shows some indication of two populations, one centered around 150 pA and the other around 350 pA.
At the later stages, the entire population is well-described by a single distribution centered around the lower value of 150 pA, with an almost complete absence of the high I_K cells.

**Development of Na\(^+\) and K\(^+\) currents in Cortical Plate and deep layer pyramidal cells**

The IZ is a continuously changing cell population, in which cells present on different embryonic days share a common state of differentiation. The data above indicate that this is reflected in relatively static physiological properties. Once these cells take up their positions in the layers of the forming cortical plate, however, this situation changes. As developmental time progresses, CP cells should continue to differentiate physiologically to gain the ability for robust repetitive firing, and to form the variety of firing types characteristic of the mature cortex (Connors and Gutnick 1990; Massengill et al. 1997). To follow the early stages of this process, we recorded from cortical cells at stages between E14 and P17, binning our data as follows: E14 (E13-14), E16 (E15-16), and E18 (E17-18) prenatally; P0 (P0-1), P2 (P2-3), P4 (P4-5), P6 (P6-7), P10 (P10-11), and P12 (P12-17) from postnatal cortex.

The same lateral region of the cortex was targeted at each stage, and cell type was noted, where possible. During embryonic stage recordings, we targeted cells in the mid-CP, with a few in the deeper parts of the developing CP. These cells are primarily cells of the early forming deeper layers, but may include cells from the subplate, or those of mid-layers still migrating through the deeper layers toward the pia. For postnatal recordings, we almost exclusively targeted pyramidal cells in the deeper layers by noting morphology
under DIC optics. This morphology was confirmed in some cases by dye filling with a biotin conjugated dextran (Fig. 4A).

The input resistance of CP cells dropped dramatically, by about a factor of 30, between E14 and P12 (Fig. 4B). This decrease occurred in two abrupt phases, one between E14 and E16 (E14: 17065 ± 4104 MΩ, n=23; E16: 6396 ± 1502 MΩ, n=20; p=0.03), and another between P2 and P6 (P2: 4901 ± 1403 MΩ, n=8; P6: 1662 ± 823 MΩ, n=18; p=0.047). Except for the high value at E14, the input resistances of CP cells in the embryonic and perinatal period (E16-P2) are the same as those found in IZ cells (5145 ± 554 MΩ; Fig. 4B, dashed line). By P12, CP input resistances have decreased by a factor of 10 from this value. This indicates that, for the most part, cells that are migrating through the IZ in the embryonic period maintain their resting membrane resistance as they enter the CP, and then subsequently acquire the low resting membrane resistance characteristic of mature cortical neurons over their first two weeks of residence in the CP.

To test whether this decrease in input resistance could simply be a function of cell growth, we measured capacitance of CP cells over the same period of development (Fig. 4C). Capacitance showed a substantial increase, beginning at about the time of birth, by a factor of about 6 (E14: 6.7 ± 0.3 pF, n=23; P10: 38.4 ± 6.8 pF, n=12; p=2.0 x 10⁻⁷). This increase in capacitance is not large enough to account completely for the decrease in input resistance, and does not exactly match its time course. Therefore, it is likely that the decrease in resistance of CP cells during this period is caused by a combination of growth and the insertion of new resting channels.

Over the same period of development, the amplitude of inward Na⁺ currents in CP cells increases by a factor of 10, from 72 ± 24 pA at E14 (n=18) to values averaging
about 800 pA between P2 and P12 (Figs. 5&7). (The apparent peak at P10 is not significantly different from values at P6 and P12). This increase is larger than the increase in capacitance over the same period, so Na⁺ current density shows a similar, though somewhat smaller, increase during this period (I_{Na} density is 14 ± 5 pA/pF at E14, n=16, and 83 ± 30 pA/pF at P6, n=13; p=0.016). Although we also measured a slight negative shift in the activation voltage of I_{Na} during this period, it is likely that at later postnatal stages, when Na⁺ currents are quite large, voltage clamp control is degraded sufficiently to make measurement of this difference unreliable. For the same reason it is possible that our values for maximal I_{Na} at later stages are under-estimates of the actual values.

Delayed K⁺ currents showed a similar pattern of increase in CP cells from E14 through P12. At E14, I_K amplitude (measured at +70 mV) was 327 ± 41 pA (n=23), and by P12 had increased by about a factor of four to 1198 ± 526 pA (n=9; p=0.012 compared to E14; Figs. 6A&7). Unlike the case for I_{Na}, however, the I_K increase was approximately the same as the increase in capacitance, so that I_K density showed no significant increase through the period from E14 to P12 (E14: 50 ± 6 pA/pF, n=22; P10 : 65 ± 18 pA/pF, n=9; p=0.31). This indicates that the increases in I_K amplitude during this period most likely reflect cell growth and the proportionate insertion of K⁺ channels. Differences in the time courses and magnitudes of I_{Na} and I_K development are also seen at earlier stages (Picken Bahrey and Moody, 2003), and thus emphasize the different developmental control of these two types of currents during early cortical development.

Pyramidal cells of mature mouse cortex often express currents activated by hyperpolarization, such as the non-selective cationic inward rectifier, I_h (Franz et al.
2000; Fields et al. 2001). Our recordings in VZ and IZ cells at embryonic stages, however, indicate that hyperpolarization-activated currents are almost completely absent in these populations (Picken Bahrey and Moody 2003; see above). In CP neurons, an $I_h$-like current appears rather abruptly near the end of the first postnatal week (Fig. 6B).

From E14 to P4, less than 10% of cells (3/41) showed any hyperpolarization-activated currents (Fig. 6B&C). In those three cells, the current we recorded activated slowly, showed inward tail currents at -60 mV, and had a mean amplitude of $38 \pm 23$ pA (measured at -120 mV). At P6, we observed a sudden increase in the number of neurons expressing this current (70%; 20/28 between P6 and P12; Fig. 6B&D), and a large increase in the mean amplitude in cells with the current ($101 \pm 25$ pA). This indicates that $I_h$ is almost exclusively a postnatal property of CP cells. Two cells at P8 showed a complete and reversible block in the presence of 5 mM cesium (a blocker of $I_h$).

It is not easy to predict how the variety of changes in voltage-clamp properties of CP cells between E14 and P17 might translate into development of firing patterns. The large increase in $I_{Na}$ accompanied by a stable $I_K$ should increase neuronal ability to generate action potentials. The large decrease in input resistance, however, might well degrade that ability. To measure firing properties directly, we recorded from CP cells at each stage under current clamp conditions, and measured their ability to generate action potentials following the termination of short depolarizing current pulses, their ability to fire repetitively during long stimuli, and the existence of spontaneous activity in the absence of applied current. These results are summarized in Figs. 8&9.

At early embryonic stages, CP cells were very limited in their ability to generate action potentials (Fig. 8 & 9A). At E14, for example, only 31% (4/13) of cells generated
an active response (defined as a positive dV/dt) after the termination of a short stimulus, a fraction only half as large as the fraction of cells with detectable Na⁺ currents measured in voltage clamp (65%). This discrepancy is probably due to the long time constant of the cells at this stage shunting the relatively rapid Na⁺ current. No cell at E14 showed spontaneous action potentials in the absence of stimuli. By the later embryonic stages, a much larger fraction of cells responded to stimuli with active responses. At E17-18, 80% (4/5) of cells were capable of generating an active response following the termination of a brief stimulus. These were broad responses with a high threshold (-21 ± 3 mV, n=7; Fig. 9B&C). Despite this change, however, no cells at any embryonic stage showed any spontaneous action potential activity in the absence of stimuli (resting potential was fairly depolarized: -40 ± 2 mV, n=34), and none were capable of firing repetitive action potentials during a 150 ms stimulus (Fig.8,E17 & 9D).

The large increases in I_{Na} amplitude that occur perinatally (see Figs. 5&7) change this situation considerably. By P0 83% of cells generated an action potential with a slightly shorter duration following the termination of a short pulse, and 2 of 8 cells tested were capable of firing repetitively during a 150 ms depolarizing stimulus. These same 2 cells also showed some spontaneous action potentials, apparently resulting from post synaptic potential ( PSP) like input on a resting potential of -54 ± 5 mV (n=14; Figs. 8,P0 & 9D). Cells at P2 appeared to be similar to P0 neurons in firing ability, with 57% (4/7) generating action potentials after a short pulse and 38% (3/8) both firing repetitively during a 150 ms pulse and generating spontaneous spikes.

Although the percentage of cells generating an action potential after a short stimulus did not change from earlier postnatal stages (66%), at P6 we saw an increase in
the fraction of cells generating repetitive action potentials during long pulses (83%). These action potentials were even shorter in duration than those at early postnatal stages (Fig. 9C). Only 25% of cells generated spontaneous action potentials, however.

At the latest stages, P10 – P12, 100% of recorded cells generated action potentials after a short stimulus at a threshold which had dropped more than 10 mV, to -34 ± 3 mV (n=12; Fig. 9B). Almost 70% of recorded pyramidal neurons were capable of repetitive firing during a long stimulus, and 65% fired spontaneously, often in response to psp-like depolarizations on an already depolarized resting potential (see Fig. 8, P10 & 9B).

Discussion

In order to begin to understand how the patterns with which voltage-gated ion channels develop in the mammalian neocortex, we have measured Na⁺, delayed K⁺, and inwardly rectifying currents in neurons of the mouse sensorimotor cortex between E13 and P17. We concentrated on migratory presumptive neurons of the Intermediate Zone from E13 to E18, and differentiating neurons of the cortical plate and deep cortical layers from E14 to P17. Our data indicate that all three types of currents, and cell input resistance, develop in different patterns during this interval.

The Intermediate Zone represents a continuously changing population of cells which have exited the cell cycle in the VZ recently, and which are migrating to take up their positions in the Cortical Plate. Therefore, IZ cells at E14 and E18, for example, should be similar in developmental state but different in both chronological age and in the cortical layer they are destined to occupy. We found that IZ cells were almost identical in their electrophysiological properties over the interval E13-E18. Cell input resistance,
capacitance, and $I_{Na}$ amplitudes did not vary significantly with stage. No IZ cells
expressed any hyperpolarization-activated currents. The only change we observed was a
steady decrease in $I_K$ amplitude of about 50% between E13 and E18. This could arise in
two ways. First, a slight decrease in $I_K$ might occur as cells migrate out of the VZ into the
IZ. This could be true only at later stages, however, since we have measured $I_K$ in the VZ
at E12, E13, and E14, and mean amplitudes are not distinguishable from $I_K$ amplitudes in
E13-E15 IZ. A progressively greater loss of $I_K$ as cells exit the VZ at later and later
stages, however, could indicate a layer-specific level of $I_K$ expression, with cells fated to
more superficial layers having smaller $I_K$ amplitudes, at least during migration. Another
possible explanation is that the presumptive inhibitory interneurons that migrate from the
ganglionic eminences into the neocortical IZ (Parnavelas et al. 2000; Anderson et al.
2001; Marin et al. 2001) express different levels of $I_K$ than do cells that arise locally in
the neocortical VZ. This explanation is consistent with our finding of two populations of
cells in the IZ defined by $I_K$ amplitude. The first population, with $I_K$ of around 150 pA,
persists from E13 – E18, and may represent the population of radially migrating neurons
from the VZ. The second population, defined by their larger $I_K$ (300 to 800 pA), is
present from E13-15, during the days of greatest migration of subpallial cells destined for
the developing cortex (Marin et al. 2001), but disappears at later stages (see Fig. 3B,D). If
this second population represents the GABAergic interneurons migrating from the
Ganglionic Eminences, their early presence and later loss could easily explain the decline
we see in $I_K$ with developmental stage. Previous findings by Hamill et al. (1991),
showing that pyramidal and non-pyramidal neurons in embryonic rat cortex express
identical Na\(^+\) current densities but different K\(^+\) currents are consistent with this possibility.

The Cortical Plate presents a different population of cells than the IZ, being composed primarily of post-migratory neurons undergoing terminal neuronal differentiation. We concentrated on the deep layers of the CP and postnatal cortex, which are formed earlier in development than the more superficial layers, allowing us to study a longer interval of development in a single population. The population is not entirely postmigratory, however, as migratory neurons destined for the more superficial layers transiently pass through those deeper layers.

We found a dramatic decrease – between 10 and 30-fold – in input resistance over the interval E14 – P12 in CP cells. This decrease occurred in two stages, one between E14 and E16, the other between P2 and P4. A portion of the drop in input resistance may be due simply to cell growth, since capacitance decreases over the same period, although by a much smaller amount. It is possible, however, that our measurement of the increase in capacitance underestimates membrane growth relative to input resistance measurements, because capacitance is measured at considerably higher frequencies, and thus may measure a smaller fraction of actual surface area in cell processes which are extending during this period. If we assume that the increase in surface area does, in fact, account only for a portion of the input resistance change, then the appearance of a new resting conductance must be responsible for the remainder of this change. A hyperpolarization-activated current (presumably I\(_h\)) appears postnatally in CP cells (Fig. 6B). Although little of this current is activated at the resting potential, the time course of its appearance is similar to the drop in input resistance and the appearance of even a small
resting conductance may have a large effect on cells which previously had a very low resting conductance. We do also see a positive shift in resting potential during the second postnatal week (see Fig. 9B), after a significant number of cells already express $I_h$, so it is possible that even a small contribution of $I_h$ with its positive reversal potential, to an initially small resting conductance could cause both the decrease in input resistance and the positive shift in resting potential.

The second major change we see in CP cells is the very large increase in amplitude of $I_{Na}$ beginning at late embryonic stages (Figs. 5&7). $I_{Na}$ increases by more than a factor of ten, much greater than the membrane area increase during the same period. It is not clear whether this represents the appearance of a new Na$^+$ channel subtype, or an increase in density of the subtype already present (Yarowsky et al. 1991; Gong et al. 1999; Zhang et al. 2001). Also beginning in the late embryonic period, there is an increase in amplitude of the delayed outward K$^+$ current, although this change is much smaller and can be accounted for simply by the increase in cell surface area. Finally, we see the appearance of a hyperpolarization-activated (inwardly rectifying) current in CP cells. This current appears relatively late in comparison to the increases in $I_{Na}$ and $I_K$. Between E14 and P4, only very few cells express this current. Between P4 and P6, there is a large increase in the fraction of cells expressing this current so that from P6 to P17, it can be detected in more than 80% of cells. This inward current activates slowly upon hyperpolarization, with very little current passing positive to -70 mV (although there was measurable current at voltages as positive as -50 mV in a few cells) and a voltage-dependent time constant of about 330 ms at -90 mV and 180 ms at -120 mV. Although we cannot definitively identify this current as $I_h$, the kinetics and voltage-
dependence are very similar to those published previously to describe $I_h$ (DiFrancesco et al. 1986; Spain et al. 1987; McCormick and Pape 1990), and block by Cs+ is a further indication that this current is $I_h$.

These complex changes in channel expression in developing CP cells have profound effects on the firing properties of the cells. In embryonic stages, CP cells show relatively low levels of excitability. When active responses can be elicited (27% of embryonic CP cells overall), they tend to be small and long in duration (Figs. 8,E14&E17 & 9C). The peaks of these responses are not sufficiently depolarized to activate a substantial fraction of the delayed K+ current, and combined with the long time constant of embryonic cells (due to their high input resistances), this results in a prolongation of the falling phase the active response so that it follows the membrane time constant (see Fig. 8,E14-center panel). None of our embryonic CP cells fired repetitively during long depolarizing stimuli. This does not, however, mean that embryonic CP cells do not fire repetitively in vivo. It is quite possible that these cells are subject to complex waveforms of depolarizing stimuli normally, and that some of these can evoke different firing patterns than we see in response to simple square-wave depolarizations.

This situation changes after the perinatal period. The combination of increasing Na+ and K+ current, decreased input resistance leading to faster time constants, and eventually, a more depolarized resting potential at the end of the second postnatal week results in the appearance of more rapidly rising and falling action potentials and repetitive firing ability. $I_h$ may also impart a slow depolarization encouraging repetitive firing at these later stages (Luthi and McCormick 1998; Moosmang et al. 1999; Bender et al. 2001). Consistent with this idea, we found that 78% of cells expressing $I_h$ generate
evoked or spontaneous AP’s compared to only 37% of cells not expressing $I_h$ ($p=0.0078$ Fisher’s exact test). An even greater difference exists between the percentage of cells with $I_h$ that generate either evoked or spontaneous repetitive activity (72%) and those without $I_h$ (7%; $p=3.5 \times 10^{-6}$, Fisher’s exact test). In addition, current clamp recordings made in the absence of stimuli indicate that a steadily increasing fraction of postnatal cells are normally firing repetitively (see Fig. 8). This latter conclusion, however, must be made with caution. Although the ability to fire repetitively clearly develops during this early postnatal period, we cannot be certain that the spontaneous activity we observe is not secondary to depolarization induced by leakage currents through the seal resistance. Although this leak should be less important in the lower resistance postnatal cells than in the high resistance embryonic cells, it still may interact with the more negative threshold to induce activity in some cases. We believe that at least some spontaneous activity is occurring normally, though, because we have recorded activity on hyperpolarized baselines and because we have observed spontaneous transient increases in intracellular $\text{Ca}^{2+}$ using optical methods in these cells in the early postnatal period (Picken Bahrey and Moody, unpublished observations).

Many of the developmental changes we have observed are summarized in Figure 10. Here, we take our data (including that of Picken Bahrey and Moody, 2003) in VZ, IZ, and CP cells between E14 and P17 to recreate a likely picture of the development of ionic currents in a single cell as it divides in the VZ, exits the cell cycle there at E14, migrates through the IZ, arrives in the CP on E16, and then undergoes later neuronal differentiation. As it proliferates, the presumptive neuron has very simple electrical properties: a high input resistance and outward $K^+$ current. Upon cell cycle exit, a second
conductance – a small Na\(^+\) current – appears and begins to increase slowly as the cell migrates. This state continues until the differentiating neuron arrives at the cortical plate. At this point, several things happen. First, the cell begins to grow rapidly, causing increases in Na\(^+\) and K\(^+\) current, and a decrease in R\(_{in}\). Na\(^+\) current and R\(_{in}\) changes are larger than can be explained by cell growth, however. Lagging slightly behind the increase in I\(_{Na}\) and I\(_{K}\), is the initial expression and increase of a third conductance, the inwardly rectifying cation current I\(_{h}\), possibly contributing to the decrease in R\(_{in}\). As the complexity of this cell increases, with expression of I\(_{h}\) and increases in Na\(^+\) and K\(^+\) conductances, the cell begins to mature electrically, gaining the ability to fire repetitively.

The changing patterns of ion channel expression we observe put some constraints on the likely generation of spontaneous activity during early cortical development. If cortical neurons generate repetitive bursts of action potentials that serve important developmental roles, then they should be most likely to do so starting in the very early postnatal period, rather than embryonically. This kind of activity has in fact been observed in the early postnatal rat neocortex (Garaschuk et al. 2000). Although the exact time of onset of such activity has not yet been determined, it is likely that the onset is limited by the ability of the individual neurons to fire repetitively. The termination of such activity, however, is probably determined by the nature of inputs to the cell or by the changing depolarizing drive supplied by conductances active at the resting potential, since the intrinsic ability to fire repetitively lasts throughout adulthood. Whatever exact role that intrinsic channel expression in individual neurons plays in timing spontaneous activity, it is clear that the changing patterns of channel expression must be taken into account in describing how developing neurons generate such activity.
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**Figure Legends**

Figure 1. Membrane Properties of IZ cells. 

- **A**: 200 µm thick slice of the embryonic day 15 lateral neocortex viewed at 40x, showing approximate locations of the VZ, IZ and CP. TuJ1 is used to stain differentiating neurons. Upper right inset shows a 4x image of E15 cortex and striatum: developing striatum is at the bottom of the figure, and the regions of lateral cortex we targeted in this study (within 200 µm dorsal to the LGE/cortical border) are boxed.

- **B**: Neurobiotin fill of a small dye-coupled cluster of IZ cells.

- **C**: Field of IZ cells under DIC optics, as we used to target IZ cells for recording.

- **D**: Input resistance of IZ cells as a function of stage (E13: 4663± 785 MΩ; E14: 4275± 500 MΩ; E15: 5436 ± 2217 MΩ; E16: 7350± 2594 MΩ; E17: 4085 ± 714 MΩ; E18: 5875± 1455 MΩ). No significant differences were found between the means at any two stages.

- **E**: IZ cell capacitance as a function of stage (E13: 5 ± 1 pF; E14: 5± 0.3 pF; E15: 6 ± 1 pF; E16: 5 ± 0.3 pF; E17: 6± 1 pF; E18: 4 ± 0.3 pF). No significant differences were found between any two stages.
Figure 2. Na⁺ current in migrating IZ cells. A: I_Na amplitude in IZ cells as a function of stage (E13: 24 ± 7 pA; E14: 58 ± 6 pA; E15: 65 ± 13 pA; E16: 79 ± 15 pA; E17: 37 ± 8 pA; E18: 44 ± 14 pA). The only significant difference is between E13 and E14-16. B: Percentage of IZ cells expressing detectable I_Na as a function of stage. The percentages of cells expressing I_Na varied, with a slight but significant decrease at the end of embryonic development (100% at E16; 67% at E18; p=0.021 by Fisher’s exact test).
Figure 3. Delayed $K^+$ current in IZ cells. 

A: The average peak $K^+$ current (measured at +70 mV) steadily declines during development in the migrating population (E13: 392 ± 62 pA; E14: 328 ± 25 pA; E15: 253 ± 37 pA; E16: 192 ± 27 pA; E17: 196 ± 40 pA; E18: 157 ± 46 pA). Histograms of $I_K$ at early, $B$, and late, $D$, embryonic stages show the loss of a population of neurons expressing high $I_K$ at later stages, while a population with smaller $I_K$ amplitudes remains steady throughout development. 

C: Activation of the outward $K^+$ current slows with development, (E13: 0.8 ± 0.1 ms; E14: 0.9 ± 0.1 ms; E15: 1.0 ± 0.1 ms; E16: 2.6 ± 1.5 ms; E17: 5.3 ± 1.4 ms; E18: 2.7 ± 1.4 ms), with significant increases in time constant between the early stages (E14 & E15) and E17 (6.9 x 10^{-10}, and 0.003, respectively). See insets in $E$. 

E: Current-voltage relationships are similar at early (E14, ●; voltage steps to -30 mV, -10 mV, 0 mV, 20 mV, 40 mV and 70 mV from a holding potential of -60 mV) and late stages (E18, ○; voltage steps to -30 mV, -10 mV, 0 mV, 20 mV, and 70 mV from a holding potential of -60 mV). The normalized current voltage relations are means from all traces at each stage.
Figure 4. Development of membrane properties of cortical plate cells. A: P2 CP neuron, recorded with Neurobiotin in the pipet. The top of the figure is toward the pia, the bottom is toward the ventricle. B: $R_{in}$ decreases with age in CP cells (E14: 17065 $\pm$ 4104 M$\Omega$; E16: 6396 $\pm$ 1502 M$\Omega$; E18: 6923 $\pm$ 1827 M$\Omega$; P0: 6000 $\pm$ 2633 M$\Omega$; P2: 4901 $\pm$ 1403 M$\Omega$; P4: 1107 $\pm$ 519 M$\Omega$; P6: 1662 $\pm$ 823 M$\Omega$; P10: 426 $\pm$ 74 M$\Omega$; P12: 524 $\pm$ 146 M$\Omega$), with significant changes between E14 and E16, and also between stages P2 and P4. Dashed line indicates mean IZ cell $R_{in}$ for comparison. C: Capacitance of these cells increases with development (E14: 6.7 $\pm$ 0.3 pF; E16: 6.4 $\pm$ 0.8 pF; E18: 9.2 $\pm$ 2.1 pF; P0: 14.6 $\pm$ 2.8 pF; P2: 25.3 $\pm$ 5.8 pF; P4: 14.6 $\pm$ 4.9 pF; P6: 18.7 $\pm$ 4.5 pF; P10: 38.4 $\pm$ 6.8 pF; P12: 32.1 $\pm$ 9.8 pF), with significant increases between E14/P0, and P0/P10.
Figure 5. Development of Na\(^+\) currents in cortical plate cells. During prenatal neurogenesis of the CP, cells express small Na\(^+\) currents (E14: 72 \(\pm\) 24 pA; E16: 104 \(\pm\) 36 pA). Perinatally, the average amplitude shows a rapid significant increase (E18: 325 \(\pm\) 89 pA; P0: 517 \(\pm\) 151 pA; P2: 782 \(\pm\) 223 pA), which levels off after P2, with postnatal averages of 750 to 850 pA. An apparent peak at P10 is not significant.
Figure 6. Development of $I_K$ and $I_h$ in cortical plate neurons. 

A: Potassium outward current amplitudes steadily increased with development, with the biggest increase occurring around the time of birth (E14: $327 \pm 41$ pA; E16: $322 \pm 47$ pA; E18: $538 \pm 153$ pA; P0: $766 \pm 167$ pA; P2: $1181 \pm 180$ pA; P4: $727 \pm 285$ pA; P6: $831 \pm 125$ pA; P10: $1353 \pm 307$ pA; P12: $1198 \pm 526$ pA). These changes paralleled increases in capacitance (see Fig. 4), so $I_K$ density remains fairly constant throughout development. 

B: Fraction of cells expressing detectable $I_h$ as a function of stage. Traces in C and D show current responses to voltage pulses to -60, -80, -100 & -120 mV from a holding potential of -60 mV for a cell at E14 (C: no $I_h$) and P10 (D: average $I_h$). 

E: $I_h$ amplitude also increased with development. Shown are mean $I_h$ amplitudes for all recorded cells $\pm$ SEM.
Figure 7. Summary records of $I_{Na}$ and $I_{K}$ in CP cells from E14 to P10. Representative current traces from voltage clamp experiments throughout embryonic and early postnatal cortical development shows a continually increasing delayed rectifier $K^+$ current and $Na^+$ current. Note that the vertical scale changes between E18 and P0. Voltage steps were from a holding potential of -60 mV to -20, -10, 10, 30 and 70 mV for E14 and E16; -30, -20, 10, 40, and 70 mV for E18; -50, -40, 0, 30, and 10 mV for P0; -40, -20, 10, 40 and 70 mV for P4; and -10, 0, 40 and 70 mV for P10.
Figure 8. Evoked and spontaneous action potential activity in developing cortical cells. Representative voltage traces show both evoked and spontaneous activity under current clamp from cortical plate cells at five stages during development. The left-most column shows voltage responses to a long current pulse, to test repetitive firing ability. The center column shows responses to a brief 4 ms stimulus, to test the ability of the cell to generate an active response after the stimulus ends. The right column shows spontaneous activity occurring in the absence of any applied current. Scaling is the same within each column. At early stages, evoked responses were small and slow, and cells never generated spontaneous activity. By P2, repetitive evoked and spontaneous activity was prevalent, and spikes were shorter in duration. These changes were even more pronounced by P10.
Fig 9. Summary of active properties of developing cortical plate cells. A: The fraction of recorded cells generating an active response after the termination of a brief stimulus increased dramatically immediately prior to birth. Total number of cells recorded are shown for each bar. “Active response” was defined by the presence of positive dV/dt after the end of the stimulus. B: Spike threshold decreased with development from -21 ± 3 mV prenatally to -34 ± 3 mV at the end of the second postnatal week (bars). Perinatally, the resting potential dropped as well (line), but subsequently became more positive, nearing threshold potential toward the end of the second week. C: Responses were broad prenatally (8.8 ± 1.3 ms), but duration steadily declined, with action potentials lasting 3.7 ± 0.6 ms at half-maximal voltage by the end of the first week. D: None of the cells we recorded during the prenatal period fired repetitively with a long stimulation. On the day of birth, however, a small percentage gained this capability. This percentage increased steadily until P12 when 80% responded with repetitive spikes. The number of cells recorded is shown for each stage.
Figure 10. Coordinated development of electrical properties in cortical cells. Based on currents we’ve recorded in this study and a previously published study (see Picken Bahrey and Moody, 2003), we’ve created a basic picture of changing electrical properties of a developing neocortical projection neuron as it progresses from proliferation through the period of synaptogenesis. As the cell is cycling, electrical properties are fairly simple: input resistance is high and a single conductance, the delayed rectifier $K^+$ current, is expressed. Upon exit from the cell cycle, the newly differentiating neuron expresses a small $Na^+$ current. This $Na^+$ current increases as the cell migrates through the IZ and eventually reaches the cortical plate. $I_K$, however, remains constant, and the cell membrane area has not increased. After reaching the cortical plate and beginning terminal differentiation, more complexity emerges. $I_K$ increases in amplitude, but not density, as the cell grows. The increase in $I_{Na}$ continues and exceeds the rate of cell growth, therefore increasing in density as well as overall amplitude. Concurrently, the input resistance of the cell falls rapidly. A new current, $I_h$, emerges toward the end of the first week; after the cell gains the ability to respond actively to inputs, and about the same time it begins to slowly but repetitively fire. During the second week in the cortex, these patterns continue, with a small further decrease in input resistance, continued cell growth and resulting $I_K$ increase, further increase of $I_{Na}$, and large increases in both $I_h$ and rate of repetitive firing.