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# Morphological and Physiological Differentiation of Purkinje Neurons in Cultures of Rat Cerebellum

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**During ontogeny, vertebrate CNS neurons differentiate from relatively simple stem cells to complex units that express unique morphological and electrophysiological characteristics. We have examined several aspects of this developmental process in an identified CNS neuronal type, the Purkinje neuron (PN) of the cerebellum. Our approach has included the use of a tissue culture preparation and immunohistochemical and electrophysiological techniques. Using immunohistochemical techniques, we have identified immature PNs in culture and examined their morphological and synaptic development. These studies have shown that (1) PNs undergo extensive morphological and synaptic development in culture, (2) the morphological characteristics of the immature PNs in culture and the developmental sequence and time course are reflective of that described for PNs *in vivo,* (3) synapse formation is initiated at an early stage of PN development in culture and proceeds concurrently with the morphological development, and (4) the main period of synapse formation is associated with the main period of dendritic development, reflecting the preferential location of synaptic sites at the dendritic region of mature PN.**

**Using electrophysiological techniques, we have examined the physiological development of PNs in culture and have correlated the stages in physiological, morphological, and synaptic development. Results from these studies show the following. (1) Mature PNs in culture exhibit complex electrophysiological properties, including the ability to generate 2 types of spike events, simple and complex spikes, and endogenously generated activity. (2) Expression of electrophysiological properties begins at an early stage in PN development, when the PNs consist of little more than a soma with a few fine perisomatic processes. (3) The earliest physiological characteristics to be expressed by the PN include sensitivity to transmitters, the ability to respond to synaptic input, and the ability to generate simple spikes. (4) Synaptic input produces spontaneous activity in young PNs, but the patterns of activity change during development as mechanisms underlying endogenously generated activity and complex spike generation are expressed, and synapse formation proceeds. (5) The ability to generate endogenous activity is established early in PN development and before the main period of synaptic and dendritic maturation; the first endogenously generated pattern to be expressed is the regular simple spike firing pattern; expression of this activity requires $Na^+$, $Ca^{2+}$, and at least 2 types of $K^+$ conductances; this pattern is exhibited by PNs with no obvious dendritic structure, suggesting that the underlying ionic mechanisms are located in the somatic region. And (6) in mature PNs voltage-sensitive ionic mechanisms are active in both the depolarizing and hyperpolarizing membrane potential ranges; during development the ionic mechanisms active in the depolarizing membrane potentials are expressed before those active in the hyperpolarizing range.**

In the vertebrate CNS the unique characteristics of different neuronal types—including their morphological structure, synaptic circuitry, and intrinsic electrophysiological properties—play a central role in establishing their physiological function. The acquisition of these characteristics occurs during the developmental period, although modifications are likely to occur throughout life. Vertebrate CNS neurons arise from relatively simple stem cells and, thus, must undergo extensive changes to reach the mature state. This maturation process has been most extensively studied on the anatomical level, where results indicate that distinct developmental stages can often be identified that reflect programmed changes in neuronal structure or circuitry (Ramon y Cajal, 1960; Jacobson, 1978). Relatively little is known about the physiological characteristics of immature CNS neurons or the timetable and sequence in the expression of mature properties. Because CNS neuronal types exhibit unique morphological and physiological properties, these questions are best studied in an identified neuronal type. However, the heterogeneity of the CNS neuronal population, the difficulty in identifying specific neuronal types in immature tissue, and the technical problems associated with recording from small, immature neurons has limited progress in this area. In the few systems where the physiological development of CNS neurons has been examined, attention was directed to the voltage-sensitive mechanisms underlying action potential generation (see Spitzer, 1979, 1984, for reviews). In these studies, the characteristics of the action potential and the underlying ionic conductances were found to change during the course of neuronal development, suggesting that the membrane proteins responsible for this aspect of neuronal excitability are expressed according to a specific developmental sequence and time course.

In addition to action potential generation, CNS neurons ex-

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hibit other electrophysiological properties that play a prominent role in establishing their unique firing patterns. Two of these properties, the ability to generate endogenous activity and the ability to generate more than one type of spike event, are exhibited by the Purkinje neuron (PN) of the cerebellum. In the PN, voltage-sensitive ionic mechanisms underlying these properties are thought to be spatially segregated between the somatic and dendritic membranes, with both the somatic and dendritic membranes being capable of active responses (Llinás and Sugimori, 1980a, b; Gruol, 1984b). For CNS neurons like the PN, which have complex membrane properties and active conductances in both the somatic and dendritic region, physiological development is likely to progress through distinct stages, as the various cellular, synaptic, membrane, and ionic components that contribute to the mature electrophysiological properties are acquired.

In the present series of experiments, our goals were to examine neuronal development of an identified vertebrate CNS neuronal type and to correlate the stages in morphological, synaptic, and physiological development. We have selected a well-characterized CNS region and neuronal type for these studies, the PN of the cerebellum. Our approach has included the use of a tissue culture preparation, which offers considerable technical advantage, and immunohistochemical and electrophysiological techniques. Our culture preparation is a modified organotypic preparation that retains some of the cellular organization of the cerebellum *in vivo* yet offers high visibility of neurons and processes, enabling a direct correlation of morphological and physiological properties. The preparation is well suited to several types of electrophysiological recording techniques and offers an opportunity to examine CNS neuronal physiology in an intact functioning system, without the use of anesthetics or acute isolation procedures that may alter or mask the phenomena of interest. Results from these studies demonstrate that during development PNs exhibit distinct stages in the expression of mature properties and that these stages are reflected in both the morphological and physiological characteristics of the neuron. Preliminary reports of these studies have been published (Franklin and Gruol, 1983, 1984; Gruol, 1983b; Gruol and Franklin, 1983a).

## Materials and Methods

*Culture methods.* Modified organotypic cultures of rat cerebellum were prepared from 20 d embryos, obtained from timed-pregnant albino Sprague-Dawley rats (Charles River, Inc.), as previously described (Gruol, 1983a).

*Immunohistochemical methods.* Two antisera were used for immunohistochemical studies: one containing an antibody directed against cyclic GMP-dependent protein kinase (cGPK; antisera used at 1:1000 dilution) and the other containing an antibody to synapsin I (antisera used at 1:5000 dilution), a protein localized to synapses (Bloom et al., 1979; De Camilli et al., 1979, 1983a, b). To immunostain the cultures, the growth medium was removed and the cultures gently rinsed with serum-free growth media (MEM). The tissue was fixed with a solution of 4% paraformaldehyde in PBS for 30 min, followed by several rinses with PBS. The incubation solution containing PBS, 0.05% Triton X-100, 0.1% BSA and the primary antibody was added, and the cultures stored overnight in the cold. On the following day, immunoreactivity was detected by an immunoperoxidase reaction using the materials and procedures provided in the Vectastain kit (Vector Laboratories). The cultures were then prepared for semipermanent storage by mounting coverslips with glycerin-jelly, a mounting medium suitable for both phase-contrast and bright-field optics. When viewed under phase-contrast optics, the cellular morphology in the stained cultures was similar to that in living cultures, making direct correlations between stained and living cultures possible. When the entire procedure was performed in the absence of primary antibody, no staining was observed.

*Electrophysiological methods.* For electrophysiological studies, the culture dish containing the cerebellar explants was transferred to a chamber attached to the stage of an inverted phase-contrast microscope and the growth medium replaced with HEPES buffered (10 mM, pH 7.3) balanced salt solution (Hank's or a similar type). A heating unit attached to the stage of the microscope maintained the recording medium at the desired temperature (22–37°C). Neurons were selected for study, and the recording and drug electrodes positioned under 400× magnification. Recordings were made from PNs, granule cells, and interneurons. All neurons studied were photographed, via a camera attached to the microscope, for later comparisons.

Two types of electrophysiological recordings were used, extracellular recordings of spontaneous action potentials (single-unit recording) and intracellular voltage recording. For all extracellular and most intracellular recordings, the patch type of recording electrode and the List EPC-7 Extracellular Patch Clamp (Medical Systems, Inc.) were used. Conventional intracellular recording techniques were used for the remaining studies. High-resolution extracellular recordings can be obtained with the patch type of electrode due to RC coupling between the recorded patch and the remainder of the neuron (Fenwick et al., 1982; Forda et al., 1982; Gruol, 1983b, 1984a, b). With this method of extracellular recording, spike shape and amplitude depend on the tightness of the seal between the electrode and cell membrane, and the on-position of the electrode relative to the active sites generating the spike events. In some extracellular recordings, baseline shifts reflecting synaptic events or slow membrane potential changes could also be recorded. An important advantage of the patch type of electrode and List clamp is that recordings can be made in different recording modes without disruption of the original recording configuration. Thus, it was possible to obtain sequential extracellular followed by intracellular recordings, an approach that was used to identify the membrane events underlying the extracellularly recorded activity and to determine if firing patterns were altered by the procedures required for intracellular recordings.

Extracellular and intracellular recordings were displayed on an oscilloscope and recorded on a polygraph for a permanent record. A ratemeter recording provided a continuous measure of firing rate and pattern. Selected recordings were stored on a 4 channel FM tape (Racal recorder) for later analysis. Spontaneous activity was characterized by visual inspection of the polygraph and ratemeter records and by computer analysis of firing rate and pattern. Computer analysis was done on-line or from tape recordings. The computer program used (developed by K. Liebold; Fish.Mac: Decus 11-320, Digital Equipment Co., Maynard, MA) creates interspike interval (ISI) histograms that provide information about firing rate, pattern, and mode of firing (single spike or burst discharge). The program keeps a continuous count of average rates and can identify and quantify aspects of the discharge pattern such as total spikes/sec, simple spikes/sec, bursts/sec, and spikes/burst. Typically, at least 1000 spike events were used to construct the ISI histograms.

Patch electrodes were made from glass hematocrit capillary tubes (Fisher Scientific) according to routine procedures (Hamill et al., 1981) and were filled with the bath saline or, when intracellular recordings were anticipated, a high-$K^+$ saline of the following composition (mM): NaCl, 8; KCl, 144; $MgCl_2$, 2; $CaCl_2$, 0.002; EGTA, 0.04; glucose, 10; HEPES buffer-NaOH (pH 7.4), 10. Electrode resistances ranged from 2 to 8 MΩ. For extracellular recordings, the electrode tip was placed close to the neuronal membrane and an electrically tight contact (seal) formed by applying gentle suction to the lumen of the electrode. Low-resistance seals (MΩ range) were adequate for extracellular recordings, although higher-quality recordings were obtained with high-resistance seals (GΩ range). For intracellular recordings the formation of high-resistance seals (GΩ range) was required. Intracellular recordings were obtained after forming a seal by rupturing the membrane patch enclosed by the electrode tip.

Fine-tipped microelectrodes filled with $K^+$-acetate (120–200 MΩ resistances) or KCl (80–130 MΩ resistances) were used for conventional intracellular recordings. A single electrode was used for both voltage recording and current injection (via a bridge circuit). For all intracellular recordings, readings of membrane potential were considered to be approximate values because of the difficulty in determining the value of the membrane potential in a spontaneously active neuron and the difficulty in assessing the correct level for the bridge balance when a single high-resistance electrode is used for both voltage recording and current injection.

The sensitivity of the PNs to the putative neurotransmitter glutamate (GLU) was tested by exogenous application by micropressure from auxiliary micropipettes (3–5 μtip) containing 50 μM GLU dissolved in the recording media. Several types of ion channel blockers were also tested: TTX (Calbiochem), $Mg^{2+}$, 4-aminopyridine (4-AP; Aldrich), and tetraethylammonium bromide (TEA; Sigma). These blockers were dissolved in recording media and applied via the perfusion system or added directly to the recording media.

## Results

*Culture characteristics and composition*

Our culture preparation is a modified organotypic system that, due to a brief trituration procedure, offers high visibility of neuronal structure yet retains some of the cellular organization of the cerebellum *in vivo*. The preparation is well suited to morphological, immunohistochemical, and electrophysiological techniques. Only the cortical region of the cerebellum is used to prepare the cultures, reducing the cellular complexity and simplifying cell identification. The cultures are prepared from 20 d rat embryos, an age at which the cerebellum is small and immature (Woodward et al., 1969a, b, 1971; Eccles, 1970; Altman, 1972, 1982; Altman and Bayer, 1978). At plating, few of the cerebellar neuronal types have developed and, due to the organotypic nature of the preparation, cellular elements cannot be clearly identified. With age in culture the tissue spreads and thins as non-neuronal cells migrate from the original explant, and by 3–5 days *in vitro* (DIV) small clusters of neurons can be identified. The neurons increase in size and complexity with culture age and eventually develop a morphology typical of "mature" cultured neurons. For large neurons such as PNs these characteristics include a large, phase-refractile soma with a centrally located, spherical nucleus containing 1 prominent nucleolus and a well-developed dendritic tree (Fig. 3). The neuronal population appears to grow on top of the larger, flat non-neuronal background cells, and for many neurons, the cellular surface is relatively free of overlying tissue.

The cultures are considered to be mature at 21 DIV based on the morphological and electrophysiological characteristics of the neurons and the fact that distinct changes are obvious before but not after this culture age. In mature cultures several neuronal types can be identified when viewed in the living state under phase-contrast optics. These neuronal types resemble the 5 neuronal types present in the cortical region of the cerebellum *in vivo* (Palay and Chan-Palay, 1974): PNs, granule cells, basket cells, stellate cells, and Golgi cells. In mature cultures, the PNs can be easily distinguished from the other cell types by their large size (the largest neuronal type in the cultures: soma length, 20–25 μm) and unique somatic and dendritic morphology (Fig. 3). The granule cells can also be clearly recognized by their small, uniform size (soma length, 8–10 μm) and fine axonal and dendritic structure, as well as the fact that they are the most numerous neuronal type in culture. The other neuronal types present in the cultures include multipolar neurons of varying size and morphology (soma length, 10–20 μm) that are immunoreactive to an antibody to GABA (Gruol and Crimi, unpublished observations) and that resemble the inhibitory interneurons of the cerebellum—the stellate, basket, and Golgi cells.

*Immunostaining of PNs in culture*

During the culture period PNs undergo extensive morphological changes as evidenced by the immature nature of the tissue at plating and the fact that by 21 d in culture the PNs exhibit many of the morphological features typical of mature PNs *in vivo*. As a first step toward defining the stages in this maturation process, the cultures were examined under phase-contrast optics at various times after plating and the morphological characteristics of the neurons noted. This approach proved useful for characterizing the growth and development of PNs in older cultures (>12 DIV) but was inadequate for young cultures because even after several days in culture the PNs could not be identified unequivocally. Consequently, immunohistochemical techniques and a protein-specific antibody were used to identify the immature PNs in culture and to develop morphological criteria for identification in the living state. The antibody used, directed against cyclic guanosine 3′:5′-monophosphate-dependent protein kinase (cGPK), an enzyme thought to play a prominent role in regulation of neuronal function (Greengard, 1981; Nestler and Greengard, 1983), selectively stains PNs in histological sections from adult rat cerebellum (Lohmann et al., 1981). The cerebellum contains large quantities of cGMP, cGPK, and the substrate for the kinase, predominately localized to the PN (Detre et al., 1984; Schlichter et al., 1980). Initially, the immunohistochemical procedures were applied to mature cultures ($n = 26$) to test the specificity of the antibody. In these cultures, neurons identified as PNs based on their unique morphology and electrophysiological properties (Gruol, 1983a) were immunoreactive, but all other cell types remained unstained (Fig. 1), consistent with the specificity of the antibody in histological sections (Lohmann et al., 1981). Immunoreactivity was observed in both the somatic and dendritic regions of the PN and appeared to be localized to the cytoplasm. Staining was most intense in the somatic region, presumably due to the greater cytoplasmic content.

The procedure was then applied to immature cultures. For these studies, the cultures ($n = 52$) were immunostained at 2 to 3 d intervals starting at 4 DIV, the earliest culture age suitable for immunohistochemical procedures (several days were required after plating for the tissue to become firmly attached to the collagen substrate). The stained cultures were examined under bright-field optics to identify the stained neurons and then under phase-contrast optics to reveal morphological details that could be used to identify the immunoreactive neuronal type when viewed in the living state. These immunohistochemical studies revealed that immunostained neurons were present at all culture ages, only one morphological class of neurons was immunoreactive, and the immunoreactive neurons exhibited distinct morphological features at all culture ages (Fig. 1). These neurons were identified as PNs based the following: (1) the specificity of the antibody for PNs in the mature cultures, (2) the specificity of the antibody for PNs in histological sections from mature and immature cerebella (Lohmann et al., 1981; Levitt et al., 1984), and (3) the similarity of the cultured PNs to published photographs and descriptions of immature PNs in histological sections from cerebella of young animals (Ramon y Cajal, 1960; Meller and Glees, 1969; Woodward et al., 1969a; Altman, 1972; Crepel et al., 1980; Hendleman and Aggerwal, 1980; Laxson and King, 1983) and in other types of cerebellar culture preparations (Hendleman and Aggerwal, 1980; Weber and Schachner, 1984). Staining for cGPK was light for PNs in 4 d cultures and increased in intensity with culture age and neuronal maturation. However, the degree of staining did not always correlate with the degree of morphological maturation, possibly because of differences in the accessibility of the PNs to

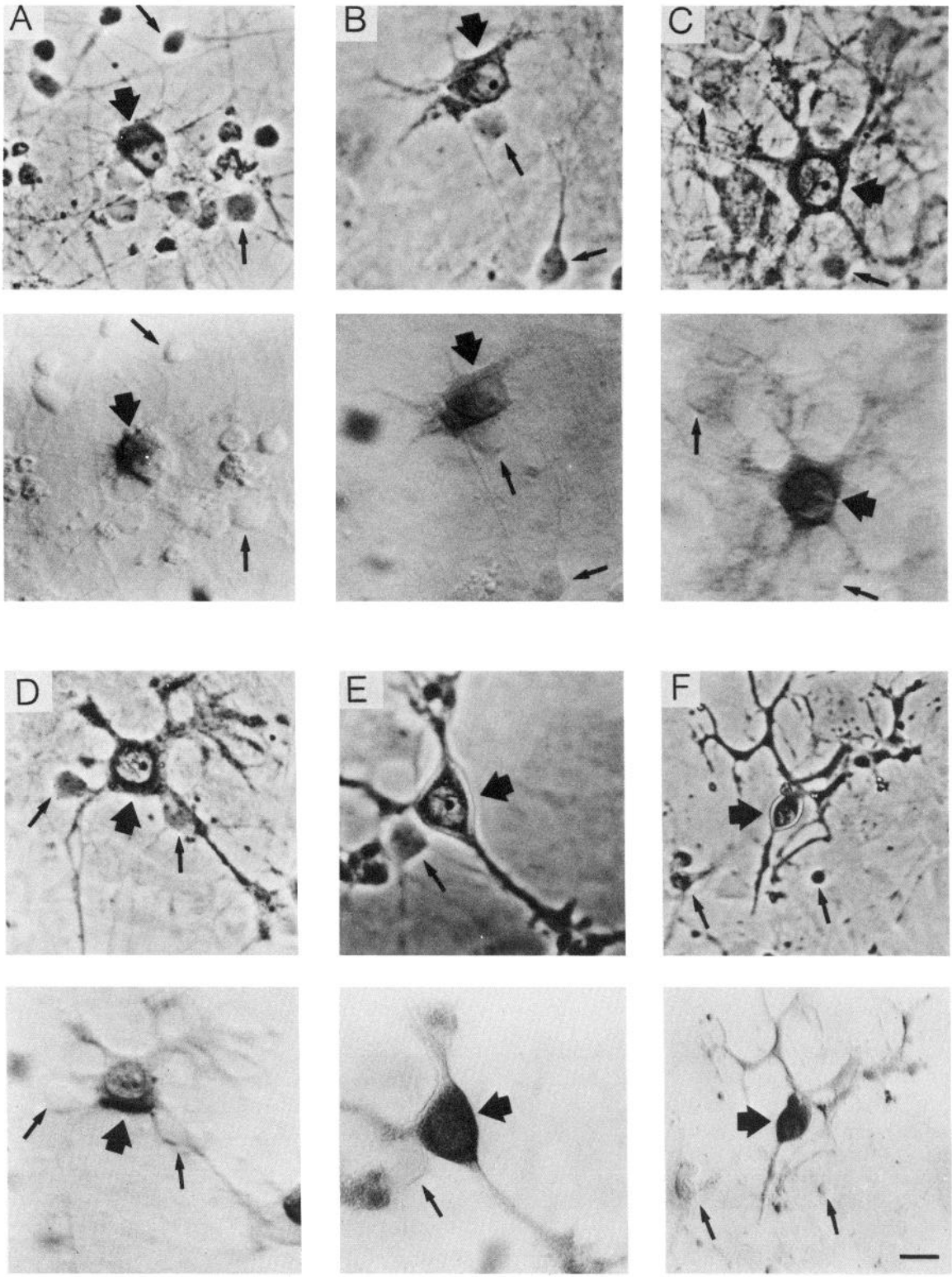


*Figure 1.* Phase-contrast (*top*) and the corresponding bright-field (*bottom*) photomicrographs of developing PNs in cerebellar cultures immunoperoxidase-stained for cGPK. Only the PNs (*large arrow*) stain with this antibody. *Small arrows* point to some unstained neurons (granule cells or inhibitory interneurons). The PNs display morphologies characteristic of the different developmental stages: *A, postneuroblast*; *B,* early *apical cone or fusiform*; *C,* late *apical cone or fusiform*; *D,* early *stellate cell with disoriented dendrites*; *E, somatic and dendritic maturation*; *F, mature.* Calibration bar, 11 μm (*A–E*), 22 μm (*F*).

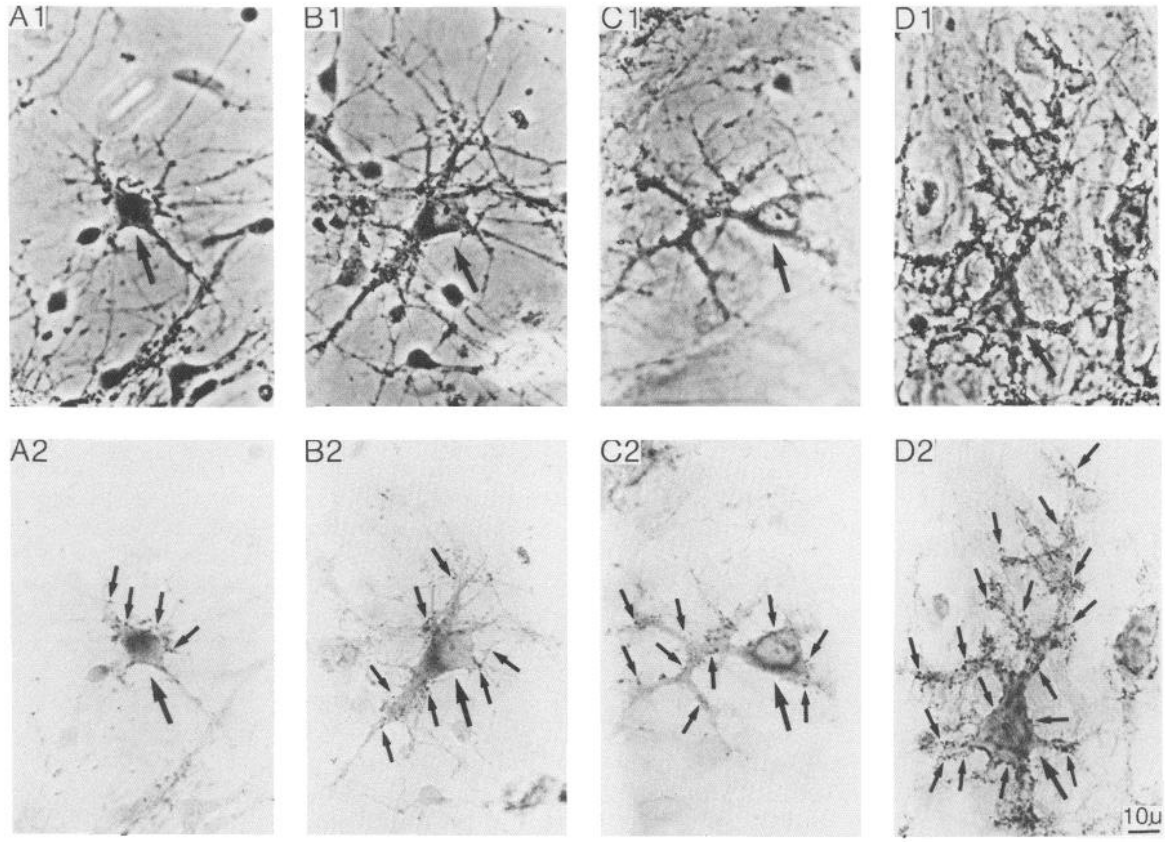


*Figure 2.* Phase-contrast (*top*) and the corresponding bright-field (*bottom*) photomicrographs of developing PNs (*large arrows*) in cerebellar cultures immunoperoxidase-stained for synapsin I. Immunostaining is punctate in nature and is considered to represent synaptic sites (a few sites are marked with *small arrows*). In young PNs (*A, B*), few synaptic sites are present, and these sites are predominately located at the base of the fine perisomatic processes. The number of synaptic sites increases with maturation. The sites are predominately located on the dendrites. The PNs display morphologies characteristic of the different developmental stages: *A* and *B, apical cone or fusiform*; *C, somatic and dendritic maturation*; *D, mature.*

the antibody (as a consequence of regional variations in cellular packing). At all culture ages the PNs were the largest neuronal type in culture.

Qualitative studies on the timing and pattern of synapse formation were also carried out using sister cultures and immunohistochemical techniques. For these studies, an antibody to synapsin I, a protein localized to synaptic vesicles was used (Bloom et al., 1979; De Camilli et al., 1979, 1983a, b). This antibody has previously been shown to stain synaptic sites in CNS tissue, including cerebellar tissue, prepared for light and electron microscopy (Bloom et al., 1979; De Camilli et al., 1983a, b). Cultures were stained at various ages after plating, starting at 6 DIV, and examined under bright-field and phase-contrast optics. Immunostaining was present at all culture ages and was punctate in nature, consistent with the type of staining observed in histological sections. Both the number of the immunoreactive sites and the diameter of the sites increased with age in culture (Fig. 2). In mature cultures, immunoreactive sites were located on both the somatic and dendritic regions of the PNs and other neuronal types. At all culture ages the immunoreactive sites were considered to reflect synaptic sites.

### *Morphological development of PNs in culture*

Examination of the immunostained cultures revealed that PNs in 4 d cultures were quite immature, consisting of a large nucleus, scant cytoplasm, and a few fine perisomatic processes. From this starting point the morphological characteristics changed dramatically as the PNs progressed through the maturation process. The early changes associated with PN differentiation included an increase in somatic volume, the sprouting of fine perisomatic processes, and the formation of synapses. Relatively few synaptic sites were present on PNs in 6 d cultures (Fig. 2). These sites were small and located on the fine perisomatic processes, either scattered along the process or clustered near the somatic region. Most of the fine perisomatic processes were temporary structures, withdrawing at later stages. However, by 8 DIV at least one could be identified as a rudimentary dendrite, being greater in diameter and length than the other processes. Dendritic development proceeded from this point, and by 2 weeks in culture, primary and secondary dendrites were well developed in most PNs (Fig. 1). Maturation of the somatic region accompanied dendritic development and consisted of an increase in somatic volume, a decrease in the number of perisomatic processes, a central positioning of the nucleus, and an accumulation of granular material (Nissl material) in the perinuclear region. An increase in the number and distribution of synapses accompanied dendritic development, with the majority of synaptic sites being localized to the emerging dendrites (Fig. 2). In mature PNs, the dendritic tree was heavily invested with synaptic sites. Synaptic sites were also present on the somatic region, but this cellular site appeared to be a less favored location. At the soma, synaptic sites were clustered, with the

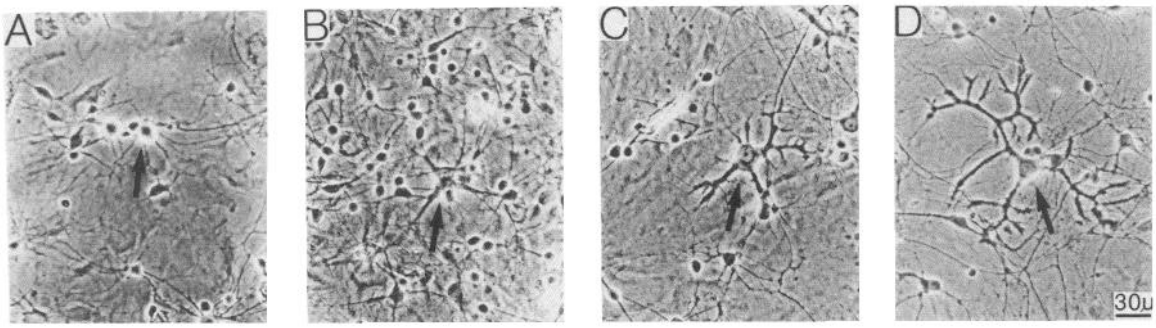


*Figure 3.* Phase-contrast micrographs of developing PNs in living cultures. These PNs exhibit characteristics typical of the following stages in morphological development: *A* and *B, apical cone or fusiform, C, stellate cell with disoriented dendrites; D, somatic and dendritic maturation.*

majority of the somatic surface being free of synaptic sites (Fig. 2).

*Developmental stages for PNs in culture*

The morphological and synaptic development of PNs in culture outlined above correlated with culture age, and at each culture age distinct profiles were observed that resembled profiles of PNs in histological sections prepared from rat cerebella of comparable age *in vivo* (Meller and Glees, 1969; Woodward et al., 1969a; Altman, 1972; Crepel et al., 1980; Hendleman and Aggerwal, 1980; Laxson and King, 1983). Based on these distinct characteristics, developmental stages could be identified for PNs in culture that correlated well with the stages described for PN development *in vivo.* As is the case *in vivo* (Altman and Bayer, 1978; Crepel et al., 1980; Inouye and Murakami, 1980), morphological development was asynchronous, and different developmental stages could often be observed in the same culture dish.

In culture, these developmental stages and the most prominent morphological characteristics were categorized (Figs. 1–3) as follows.

*1. Postneuroblast* (0–4 d cultures). The PNs are small and rather undifferentiated. The nucleus is rounded, eccentrically located, and fills most of the somatic region. Fine perisomatic processes are starting to emerge from the somatic region.

*2. Apical cone or fusiform stage* (4–9 d cultures). The cell body is rounded or elongated with many thin, branching perisomatic processes. Larger outgrowing processes, considered to be dendrites, are starting to emerge from the somatic region. The dendrites have tufts of fine branches on their tips. The nucleus is large and rounded, contains 1 large nucleolus, and is eccentrically located. Synaptic sites are present on the soma and fine perisomatic processes.

*3. Stellate cells with disoriented dendrites* (8–15 d cultures). The cell body is elongated, triangular, or stellate shaped. The nucleus is more centrally located. The number of fine, perisomatic processes is reduced. Several large processes with branching tips, considered to be dendrites, emerge from the somatic region. The dendrites have both primary and secondary branches and are irregular in contour. Synaptic sites are present, predominately on the dendrites.

*4. Somatic and dendritic differentiation* (13–20 d cultures). The soma is large and rounded or pear-shaped. The nucleus is centrally located, and Nissl material is evident in the perinuclear

**Table 1. Changes in the extracellularly recorded activity of PNs with development in culture**

| Culture age (d) | $n^a$ | Spontaneously active PNs | Firing rate (Hz) | | Predominant spike event[b] | | | Predominant firing pattern[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | Range | SS | DS | CS | Reg. | Inter. | Irreg. |
| 4 | 46 | 10 | 1.7 | <1–5 | 10 | 0 | 0 | 3 | 1 | 6 |
| 6 | 49 | 46 | 3.0 | 1–7 | 38 | 6 | 2 | 20 | 8 | 18 |
| 7 | 13 | 13 | 2.5 | 1–4 | 13 | 0 | 0 | 5 | 2 | 6 |
| 8 | 16 | 16 | 3.5 | 1–5 | 16 | 0 | 0 | 7 | 4 | 5 |
| 9 | 22 | 22 | 4.9 | 2–16 | 20 | 1 | 1 | 12 | 4 | 6 |
| 13 | 10 | 10 | 7.3 | 5–12 | 10 | 0 | 0 | 8 | 1 | 1 |
| 14 | 16 | 16 | 6.8 | 3–15 | 13 | 1 | 2 | 10 | 4 | 2 |
| 15 | 4 | 4 | 2.3 | 2–3 | 4 | 0 | 0 | 3 | 1 | 0 |
| 16 | 26 | 26 | 6.7 | 1–12 | 16 | 0 | 10 | 13 | 5 | 8 |
| 17 | 29 | 29 | 8.7 | 4–15 | 24 | 1 | 4 | 18 | 4 | 7 |
| 20 | 3 | 3 | 8.0 | 8 | 3 | 0 | 0 | 1 | 0 | 2 |
| >20 | 97 | 97 | 13.0 | 2–29 | 56 | 19[d] | 22 | 84 | 5 | 8 |

[a] $n$ = number of PNs tested.

[b] Number of PNs in which the predominant spike events were simple spikes (SS), doublet spikes (DS), or complex spikes (CS).

[c] Number of PNs showing regular patterns (Reg.), intermittent patterns (Inter.), or irregular patterns (Irreg.).

[d] Includes 5 PNs firing in burst of 3–4 SS spikes rather than 2 SS as is defined for the doublet.

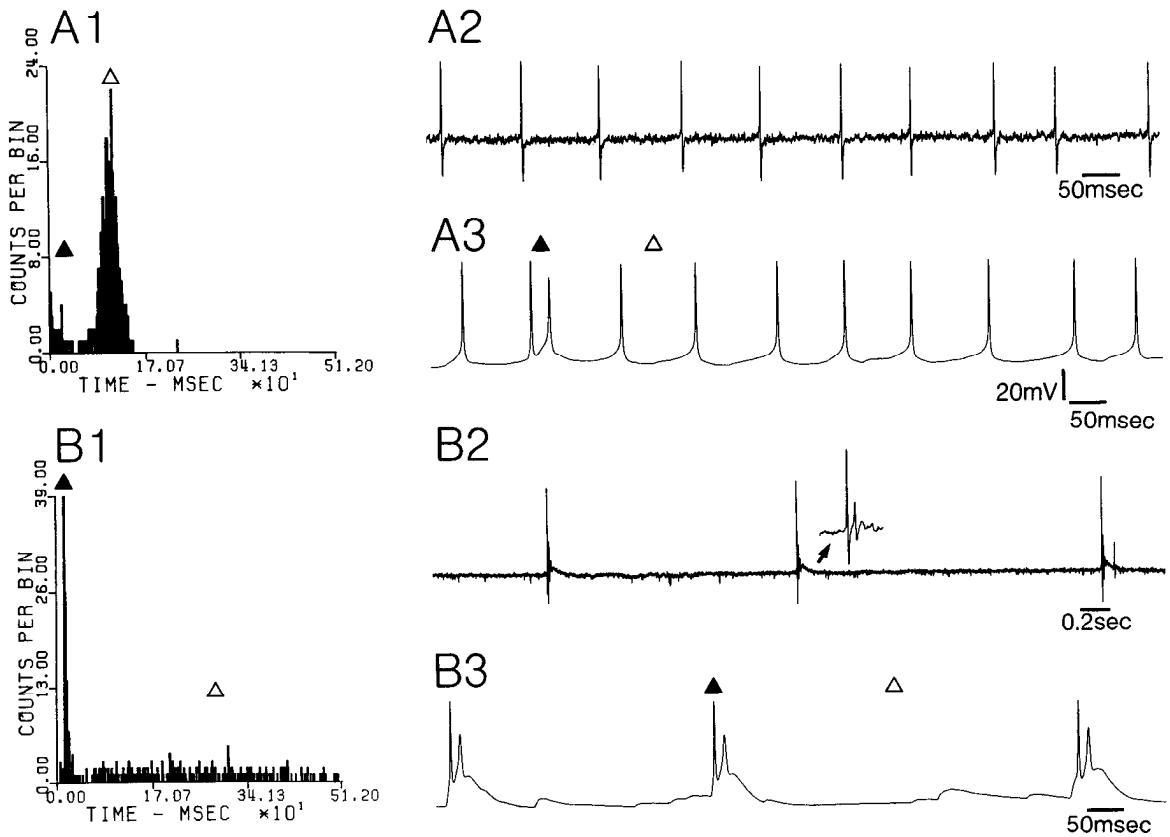


*Figure 4.* Extracellular (*A2, B2*) and subsequent intracellular (*A3, B3*) recording from PNs in mature culture exhibiting common firing patterns. The ISI histograms constructed from the extracellular recordings are shown in *A1* and *B1*. The interspike intervals marked with *triangles* on the recording indicate the intervals generating the similarily marked peaks in the histograms. The PN in *A* exhibited a regular simple spike firing pattern. This pattern is endogenously generated by pacemaker potentials that evoke simple spikes. Synaptic input also contributes to the pattern and is thought to generate the shorter interval events (*closed triangle*) and early peak in the ISI histogram. Synaptic input, more clearly seen at more hyperpolarized membrane potentials, occurs at irregular intervals and cannot account for the regular firing pattern. The PN in *B* exhibited an irregular pattern of complex spikes. In the intracellular recording, the complex spike response was identified as arising from large membrane depolarizations. The irregular nature of the complex pattern in this PN and the presence of synaptic potentials in the recording suggest that synaptic input generated the complex spikes in this PN. Recordings made with patch electrodes.

region. Most of the perisomatic processes have disappeared, and the number of dendrites emerging from the soma is reduced. The dendritic tree is well developed. The dendrites are thick and more regular in shape. Many synaptic sites are present.

*5. Mature morphology* (21 d and older cultures). The somatic region is large and rounded or pear-shaped. The nucleus is large, spherical, and centrally located with 1 prominent nucleolus. Nissl material is prominent. The dendritic tree is well developed with large branching processes and dendritic spines. The dendritic tree is heavily invested with synaptic sites. Synaptic sites are also present on the somatic region and are clustered.

### *Electrophysiological properties of mature PNs in culture: extracellular studies*

Extracellular recordings from morphologically mature PNs were used to characterize the physiological properties of mature PNs in culture and to compare properties expressed in culture to that described for PNs *in vivo.* Recording procedures and data analysis were similar to those used for *in vivo* studies, enabling a direct comparison of results. *In vivo,* PNs exhibit spontaneous activity in distinct firing patterns generated by 2 types of spike events, simple spikes and complex spikes (Eccles et al., 1967; Bell and Grimm, 1969; Woodward, 1969b; Lathan and Paul, 1971; Martinez et al., 1971; Llinás, 1981; Ito, 1984). Simple spikes are short-duration, biphasic action potentials of uniform amplitude and duration; complex spikes are multiphasic events characterized by bursts of 2–5 action potentials of decreasing amplitude and increasing duration, followed by a period of inhibition. Activity patterns vary among PNs *in vivo,* ranging from a constant regular simple spike pattern, at rates of 25–50 Hz, to intermittent, phasic or irregular patterns. Complex spikes, occurring fairly regularly at 1–2/sec, are superimposed on the ongoing pattern.

Extracellular recordings revealed that mature PNs in culture ($n$ = 97) exhibit spontaneous activity and that firing patterns and spike events are similar to those characteristic of mature PNs *in vivo.* The spike events exhibited include simple spikes, identified by the similarity to simple spikes *in vivo,* burst discharges similar to complex spikes, and a doublet event characterized as a burst of 2 simple spikes of similar amplitude and duration. Typically, more than 1 type of spike event contributed to the patterns of activity. Patterns could be divided into 3 categories based on the predominant spike event and regularity of the activity (Figs. 4, 5): (1) regular or intermittent patterns of simple spikes, (2) regular or intermittent patterns of doublet or complex spikes, and (3) irregular and usually mixed patterns of simple, doublet, or complex spikes. Intermittent patterns were produced by pauses in the regular firing patterns. These different

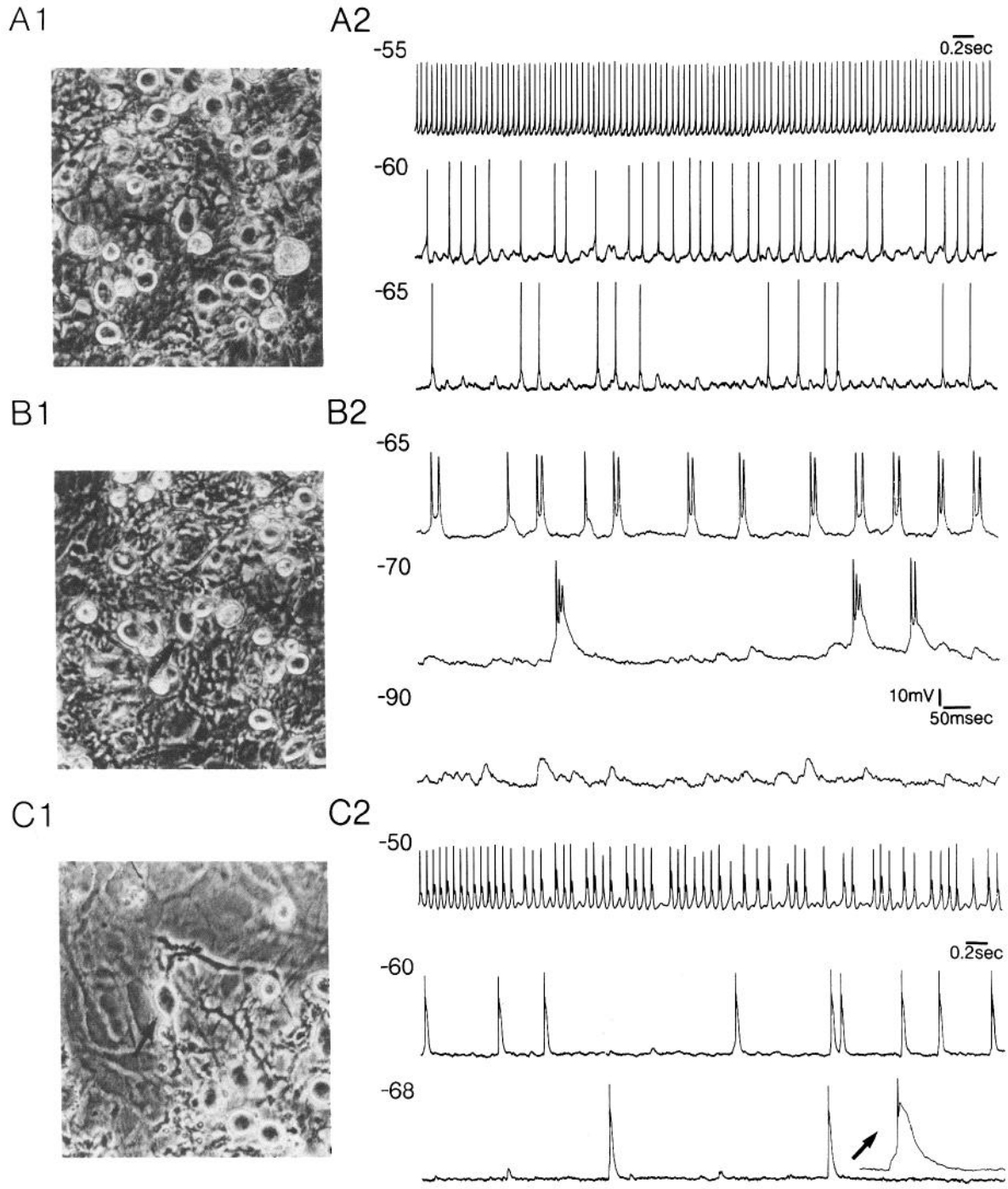


*Figure 5.* Intracellular recordings illustrating the types of spontaneous activity exhibited by mature PNs in culture and the effect of membrane potential on the firing patterns. The PNs are shown in the phase-contrast micrographs at *left* (*arrow*). The PNs in *A* and *B* are from the same culture (80 DIV). The PN in *A* exhibited a regular simple spike firing pattern; the PN in *B* exhibited a mixed pattern of doublet and simple spikes; and the PN in *C* (40 DIV) exhibited a pattern of complex spikes. For all 3 types of activity, firing rate decreased in a membrane potential-dependent manner when the membrane potential was hyperpolarized by intracellular current injection. Synaptic input and endogenously generated activity contributed to all 3 patterns of activity. The synaptic events can be seen at the most hyperpolarized membrane potential for each PN. Conventional intracellular recording techniques were used. $K^+$-acetate-filled electrodes.

firing patterns produced characteristic ISI histograms, making it possible to identify firing patterns by inspection of the histogram. Regular patterns of activity produced prominent peaks at the modal interval, the tightness of the peak reflecting the degree of regularity of the activity; irregular patterns produced broad histograms. The most common firing pattern observed in the mature PNs was a regular simple spike firing pattern similar to that observed for PNs *in vivo* (Fig. 4*A*, Table 1). These data indicate that many of the electrical and chemical mechanisms that underlie PN activity *in vivo* are expressed in culture, under conditions in which differentiation occurs during the culture period, and support the validity of the culture preparation as a model system for studies of neuronal development at the cellular level.

*Electrophysiological properties of mature PNs in culture: intracellular studies*

Intracellular recordings were used to identify the membrane events and cellular mechanisms generating the patterns of activity observed in the extracellular recordings and to examine the membrane properties of the mature cultured PN in more detail. Initially, sequential extracellular followed by intracellular recordings were made, so that the activity recorded extracellularly could be directly correlated with the underlying membrane events. In these studies, the patterns of activity and spike events recorded intracellularly correlated well with the extracellularly recorded activity, suggesting that the procedures used to obtain the intracellular recording did not significantly damage the PN. These studies revealed that the simple, doublet, and complex spike recorded extracellularly are associated with unique membrane events (Fig. 4). The simple spike is a rapid membrane event consisting of a depolarization immediately followed by a pronounced hyperpolarization. In contrast, the complex spike is a prolonged event consisting of a large membrane depolarization and multiple spikes of differing amplitude and duration. Doublet events are 2 simple spikes in close succession superimposed on a small membrane depolarization (Fig. 5).

As in the extracellular recordings, firing patterns and spike events recorded intracellularly varied among PNs (Figs. 4, 5). Consistent with previous studies, synaptic events and endogenously generated activity contributed to the patterns of activity (Gruol, 1983a). To assess the contribution of these 2 cellular mechanisms to the firing patterns observed in the present study, 2 types of experiments were carried out: (1) The membrane properties of PNs exhibiting different firing patterns were examined to determine if differences in firing pattern were related to differences in membrane properties, and (2) extracellular recordings were made from the granule cells and inhibitory interneurons to characterize their firing patterns and, consequently, the expected pattern of synaptic input to the PNs. Membrane properties were characterized by the type of voltage responses evoked by depolarizing current pulses and the effect of membrane potential on spike events and activity patterns.

In PNs exhibiting only simple spikes or mixed patterns of simple and complex spikes, depolarizing current pulses evoked repetitive simple spikes of the same or decreasing amplitude; in PNs where complex spikes were the predominant spike event, depolarizing current pulses evoked a complex spike (data not shown). These results indicate that the ability to generate simple or complex spikes is a property of the PN membrane and varies among PNs. The fact that complex spikes were not evoked by depolarizing current pulses in PNs where both simple and complex spikes contribute to the spontaneous activity suggests that the complex spikes were not initiated in the somatic region in these PNs (where the electrode is located) but at an extrasomatic site. This interpretation is consistent with the proposal that complex spikes are initiated in the dendritic region (Eccles et al., 1967; Ekerot and Oscarsson, 1980; Llinás and Sugimori, 1980b; Crepel et al., 1981; Llinás, 1981).

In PNs exhibiting regular patterns of activity (simple, doublet, or complex spikes), the spike events were associated with pacemaker potentials, and the patterns were highly sensitive to membrane potential, suggesting that intrinsic voltage-sensitive mechanisms played a major role in establishing these patterns of activity (Figs. 5, 6). Thus, when the membrane potential was hyperpolarized, the frequency of spontaneous events decreased in a membrane potential-dependent manner, and the regular patterns were converted to intermittent or irregular patterns without the appearance of regular synaptic events that would be indicative of generation via synaptic mechanisms. Alterations in membrane potential also changed the type of spike event generated in some PNs. In these PNs, the regular simple spike pattern observed at resting membrane potential was changed to a doublet pattern by hyperpolarizing the membrane potential and then, with further polarization, to a complex spike pattern (Fig. 6). These transitions in the type of spike generated were reflected in the type of membrane response evoked by depolarizing current pulses at each membrane potential tested, with the current-evoked spike exhibiting the same form as the spontaneous spike.

These results show that mature PNs have complex membrane properties in both the depolarizing and hyperpolarizing membrane potential ranges and that the complex properties are due to the presence of a variety of voltage-sensitive ionic mechanisms. These mechanisms produce 3 types of spike events (simple, complex, and doublet) and pacemaker potentials to sustain the spiking activity; these mechanisms also render the firing patterns highly dependent on the membrane potential. Complex spikes were observed at resting membrane potential in some PNs but only at hyperpolarized membrane potentials in others, suggesting either that more than one mechanism generates this type of membrane response or that the mechanism is located in both the somatic and dendritic regions.

In preliminary studies, the effect of ion channel blockers on the endogenously generated activity of mature PNs was tested to identify the classes of ion channels required for expression of this property. These studies indicate that at least 3 classes of voltage-sensitive ion channels are required to express endogenously generated activity, $Na^+$, $Ca^{2+}$, and $K^+$. Thus, the $Na^+$ channel blocker TTX (1 $\mu$M) and the $Ca^{2+}$ channel blocker $Mg^{2+}$ (10 mM) completely blocked all spontaneous activity, whereas the $K^+$ channel blockers TEA, at concentrations that block the $Ca^{2+}$-activated $K^+$ channel (1 mM; $K^+_{Ca}$; Gruol, 1984b), and 4-AP, at concentrations known to block the A-current (0.5 mM; $K^+_A$; Gustafsson et al., 1982), significantly altered spike shape and patterns of activity (data not shown).

Synaptic input also contributed to the spontaneous activity of PNs and could be most clearly identified at the hyperpolarized membrane potentials where the endogenously generated activity was not prominent. Both excitatory and inhibitory synaptic potentials were recorded in all mature PNs studied and were identified as those potentials that were less than 100 msec in duration, had a relatively rapid rise time, varied in amplitude and polarity when the membrane potential was altered, and occurred at irregular intervals (Fig. 5). Unlike the endogenously generated activity, synaptic events occurred in slow (1–3 Hz), irregular patterns. Extracellular recordings from the granule cells and inhibitory interneurons, identified by morphological criteria, revealed that these neuronal types are silent or displayed slow, irregular patterns of activity, consistent with the characteristics of the synaptic activity observed in the PNs (data not shown).

*Electrophysiological properties of immature PNs in culture: extracellular studies*

Electrophysiological recordings were made from developing PNs in 4–20 d cultures to characterize the physiological properties of immature PNs and to identify the time course and sequence in the expression of the mature electrophysiological properties

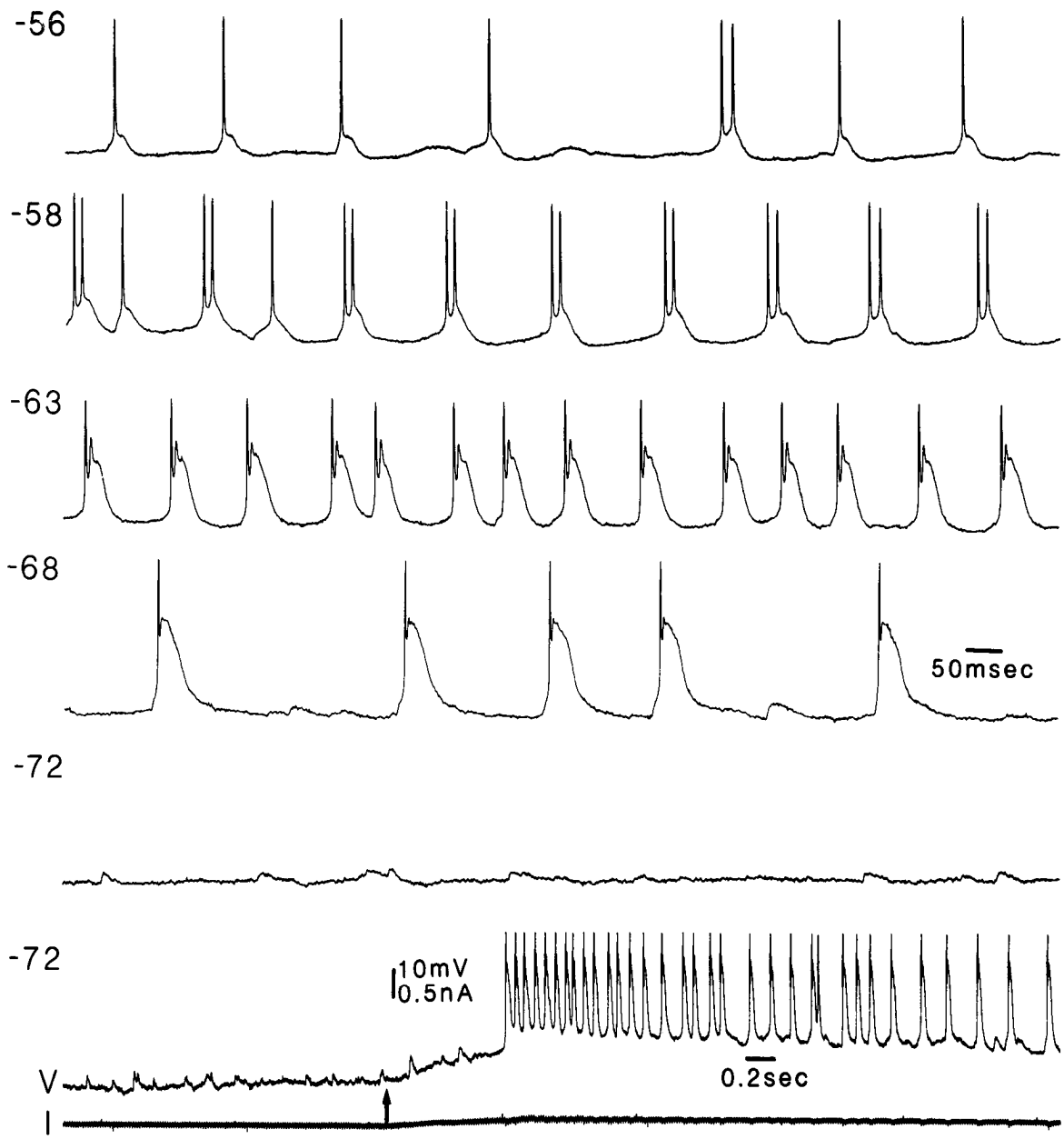


*Figure 6.* Intracellular recording from a mature PN (87 DIV) showing the complex membrane physiology exhibited by many PNs. In this PN, endogenously generated activity was the main determinant of the activity pattern. Relatively small changes in membrane potential significantly altered the type of spike generated and the final pattern. This PN could generate all 3 types of spike events, simple, doublet, and complex spike, depending on membrane potential. The contribution of synaptic input to the spontaneous activity is seen when the membrane potential was hyperpolarized to a potential (−72 mV), where the voltage-sensitive mechanisms underlying the endogenous activity were not active. In the *bottom trace,* the holding current (*I*) was reduced, depolarizing the membrane potential (*V*) and activating the mechanisms underlying the endogenously generated activity. Conventional intracellular recording techniques were used. $K^+$-acetate-filled electrodes.

described above. PNs exhibiting distinct features representative of the stages in morphological development were selected for study. Because of the small size of the immature neurons and their susceptibility to damage with intracellular recordings, extracellular recordings were emphasized. Spontaneously active PNs were present at all culture ages, although not all PNs were spontaneously active in the young cultures. Except for the youngest culture ages, most PNs exhibited more than 1 type of spike event (simple spike, doublet, or complex spike). As for mature PNs, firing patterns could be characterized by the predominant spike event, regularity of the firing pattern, and ISI histogram. Both firing rate and firing pattern changed during the maturation process.

At 4 DIV the PNs studied ($n$ = 46) were classified as *early apical cone or fusiform stage* in terms of their morphological development. The majority were silent, consistent with an early stage in both morphological and electrophysiological development (Table 1). However, GLU could evoke simple spikes in many of the silent PNs (17 responding/39 tested), indicating that the chemical and ionic mechanisms responsible for the sensitivity to this transmitter, and the voltage-sensitive ionic mechanisms underlying action potential generation, had already

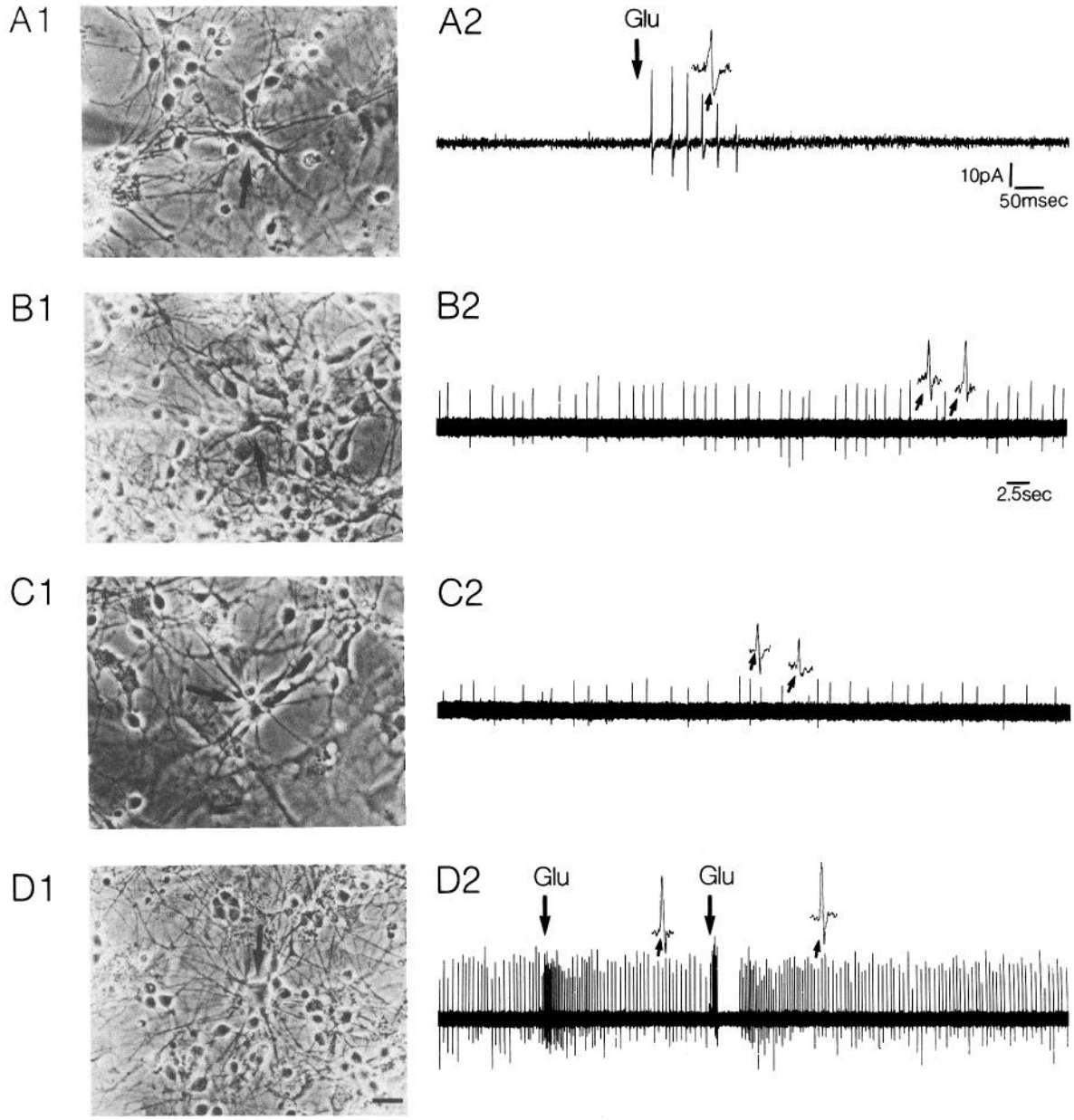


*Figure 7.* Extracellular recordings from immature PNs in 4 d cultures. The recorded neurons (*arrows*) are shown in the phase-contrast micrographs at *left* (*A1–D1*) and sample recordings at *right* (*A2–D2*). These neurons were classified as early *apical cone or fusiform* in terms of their morphological development. The PN in *A* was not spontaneously active but could generate simple spikes when the excitatory transmitter glutamate (*Glu*) was applied. The other 3 PNs exhibited slow patterns of simple spikes. The PN in *D* exhibited a regular pattern of simple spikes, similar to that observed in mature PNs but at a slower firing rate. In spontaneously active PNs, Glu evoked an increase in simple spike firing or a complex response consisting of increased simple spike activity and periods of pauses (*D2*). All recordings are AC coupled.

been expressed in many of the silent PNs (Fig. 7). The GLU response consisted of a dose-dependent burst of simple spikes associated with a baseline shift suggestive of a change in membrane potential. The later spikes of the burst were usually smaller in amplitude and longer in duration than the initial spikes, consistent with a membrane depolarization mediating the reponse.

Spontaneous activity was observed in 10 of the 46 PNs studied in 4 d cultures, the predominant form being a slow ($\leq$1 Hz), irregular pattern of simple spikes (Fig. 7). Intracellular recordings demonstrated that this pattern was synaptically evoked (see below). Surprisingly, at this early age, when the PNs were morphologically quite immature, 3 PNs displayed a regular simple spike firing pattern, similar to, but at a lower frequency than, the endogenously generated activity characteristic of mature PNs, suggesting an early stage in the development of the mechanisms responsible for this characteristic firing pattern (Fig. 7*D*). Spontaneously active PNs were also sensitive to GLU. In these PNs, the GLU response consisted of an increase in simple spike firing or a biphasic increase followed by a period of inhibition (Fig. 7*D*).

The majority of PNs studied in 6–8 d cultures and many of the PNs in 9 d cultures were classified as *apical cone or fusiform stage* in terms of their morphological development. The re-

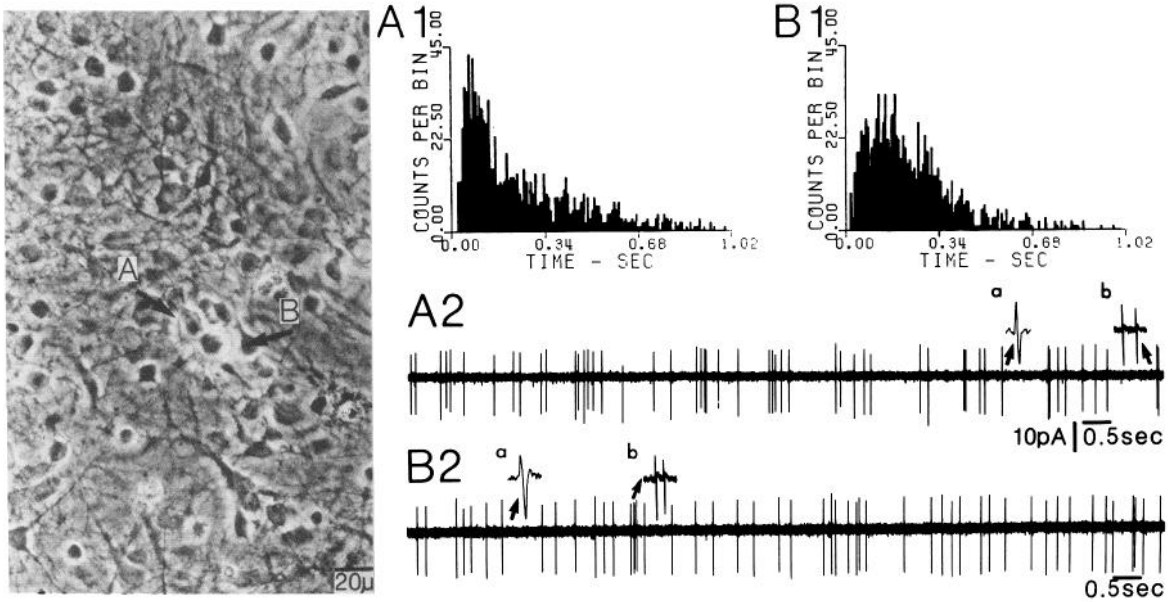


*Figure 8.* Extracellular recordings (*A2, B2*) from 2 immature PNs in a 6 d culture that exhibited firing patterns common for this stage in development. The 2 neurons (labeled *A* and *B*) are shown in the phase-contrast micrograph at *left*. Both PNs were classified as *apical cone or fusiform* in terms of their morphological development. Both PNs exhibited an irregular pattern of simple spikes and doublets (insert *b* in records). *Inserts* show the spike events at a faster time base. The corresponding ISI histograms are shown in *A1* and *B1*. The broad peaks are characteristic of irregular patterns of activity. Firing rate was 4 Hz for both PNs. Recordings are AC coupled.

maining PNs studied had progressed to the *stellate cell with disoriented dendrites stage,* where dendritic structure was clearly evident. However, firing pattern did not correlate with dendritic development, suggesting that ionic mechanisms located in the dendritic region were not a prime factor in defining the firing patterns at these ages. In the 6 d cultures all but 3 of the PNs studied ($n = 49$) were spontaneously active and all 3 of the silent PNs generated simple spikes in response to GLU. In the spontaneously active PNs, 2 types of spike events were observed, simple spikes and doublets. The predominant firing patterns were a slow, fairly regular simple spike pattern or an irregular pattern of simple spikes and doublets (Fig. 8, Table 1). Similar results were obtained in 7–9 d cultures, where all PNs studied were spontaneously active ($n = 51$). However, at these ages the main type of spike event was the simple spike, with doublet spikes being less common than at 6 DIV. The predominant firing mode in 7–9 d cultures was a regular or intermittent pattern of simple spikes (Table 1, Fig. 9).

In older cultures (13–20 d), the PNs studied ($n = 58$) exhibited morphologies typical of the *somatic and dendritic differentiation* or *mature* stage of morphological development, and all were spontaneously active. All 3 types of spike events characteristic of mature cultured PNs (simple, doublet, complex) were observed at these culture ages, with regular patterns of simple or complex spikes being the most frequent firing modes (Fig. 10). These patterns of activity were similar to patterns observed in mature PNs, but firing rates were lower, suggesting that the underlying mechanisms were in the final stages of development. Variations in the basic firing pattern were more common at these older ages and consisted of pauses or irregularly occurring spike events. These irregular spike events presumably originated from synaptic input, which is much more extensive in the older PNs. Exogenous application of GLU evoked a prolonged, multiphasic response similar to that observed in mature PNs, suggesting that the mechanisms associated with synaptic transmission were also in the final stages of development (Fig. 11).

Results from these developmental studies demonstrate that expression of electrophysiological properties in the PN is initiated by 4 DIV and that the earliest characteristics to be expressed include sensitivity to the excitatory transmitter GLU and the ability to generate simple spikes. From this starting point, 3 transitions occur in the acquisition of mature electrophysiological properties. The first transition occurs between 4 and 6 DIV, when the capability to generate spontaneous activity develops, and the second transition occurs between 6 and 9 DIV, when early patterns of spontaneous activity are converted to regular patterns of simple spikes. These 2 transitions are not dependent on pronounced changes in morphological characteristics of the PN. The third transition occurs between 9 and 13 DIV, a period of extensive morphological development, and results in the expression of complex spikes.

As a first step toward identifying the ionic conductances underlying the activity patterns, spike events and developmental changes outlined above, the effect of several ion channel blockers on the spontaneous activity of immature PNs was tested. As for mature PNs, TTX (1 μM) or $Mg^{2+}$ (10 mM) blocked all spontaneous activity in the immature PNs (6–17 DIV). TEA, at concentrations that block $K^+_{Ca}$, and 4-AP, at concentrations known to block the $K^+_A$ (0.5 mM), significantly altered spike shape and firing pattern (Fig. 12). However, the effect of 4-AP on spontaneous activity was more pronounced in the younger PNs (compare Figs. 12, 13). These data indicate that several types of ion channels known to mediate activity of mature PNs—including $Na^+$, $Ca^{2+}$, $K^+_{Ca}$, and $K^+_A$—are expressed early in development and contribute to the early firing patterns of the immature PNs. However, the mature state is not reached until

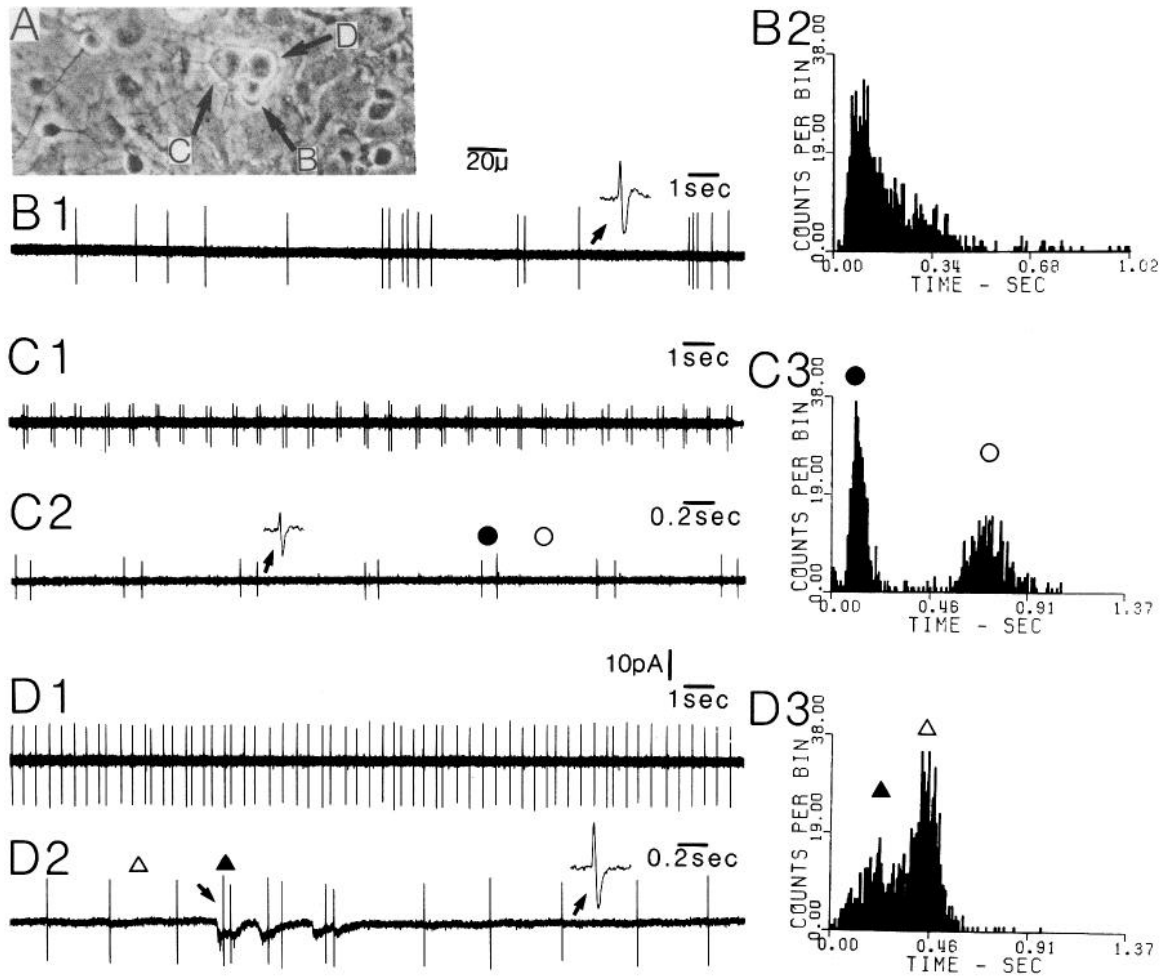


*Figure 9.* Extracellular recordings from 3 neurons in a 9 d culture that exhibited firing patterns common for this stage in development. The neurons are shown in the phase-contrast micrograph (*A*). Neuron *B* was identified as an interneuron based on morphological criteria. Neurons *C* and *D* were PNs. Both PNs were classified as *apical cone or fusiform* in terms of their morphological development. Sample recordings are shown in *B1, C1, C2, D1, D2* and corresponding ISI histograms in *B2, C3, D3*. The ISI marked with symbols on the recording indicate the intervals generating the similarly marked peaks in the histograms. Interneuron *B* exhibited an irregular pattern of simple spikes. The PN labeled *C* exhibited a regular pattern of doublets (shown at 2 time bases: *C1, C2*). The PN labeled *D* exhibited a regular pattern of simple spikes (*D1*) and intermittent events (*arrow, D2*) associated with baseline shifts thought to reflect synaptic potentials. The synaptically evoked activity (*triangle*) contributes to the early peak of the ISI histogram. Recordings *B1, C1, C2, D1* are AC coupled; *D2* is a DC recording.

the later stages of development, as evidenced by the lower firing rate of the younger PNs, the difference in type of spikes generated, and the response to 4-AP.

*Electrophysiological properties of immature PNs in culture: intracellular studies*

Intracellular recordings were made from PNs in 4–20 d cultures to identify the membrane events and mechanisms responsible for the activity observed extracellularly. These studies revealed that, at the membrane level, spike events and the firing patterns recorded in immature PNs were similar to those observed in mature PNs and that both synaptic and endogenous voltage-sensitive mechanisms also contributed to the activity patterns characteristic of immature PNs (Figs. 14, 15). In the youngest PNs studied (4 DIV), synaptic potentials were present and produced slow irregular patterns of simple spikes, reflective of the activity patterns recorded extracellularly. Depolarizing current pulses evoked single or repetitive simple spikes. By 6–8 DIV, both synaptically evoked and endogenously generated activity could be identified. The extent of synaptic input varied among the PNs but usually consisted of irregular bursts of synaptic potentials (Fig. 15). Synaptic events evoked both simple and doublet spikes and produced irregular patterns of activity. At these ages, the endogenously generated activity consisted of pacemaker potentials that evoked simple or doublet spikes and produced regular or intermittent patterns of activity. Spontaneous complex spikes were not observed. As for mature PNs, the spontaneous activity was sensitive to membrane potential. The frequency and pattern of spontaneous activity were altered as the membrane potential was varied; hyperpolarizing membrane potentials could change the firing pattern from simple spikes to doublets, as occurs in mature PNs, but complex spikes were not observed (Figs. 6, 14). However, depolarization of the membrane by intracellular current pulses could evoke a slow membrane response and repetitive spiking, suggestive of an early stage in the development of the voltage-sensitive mechanisms

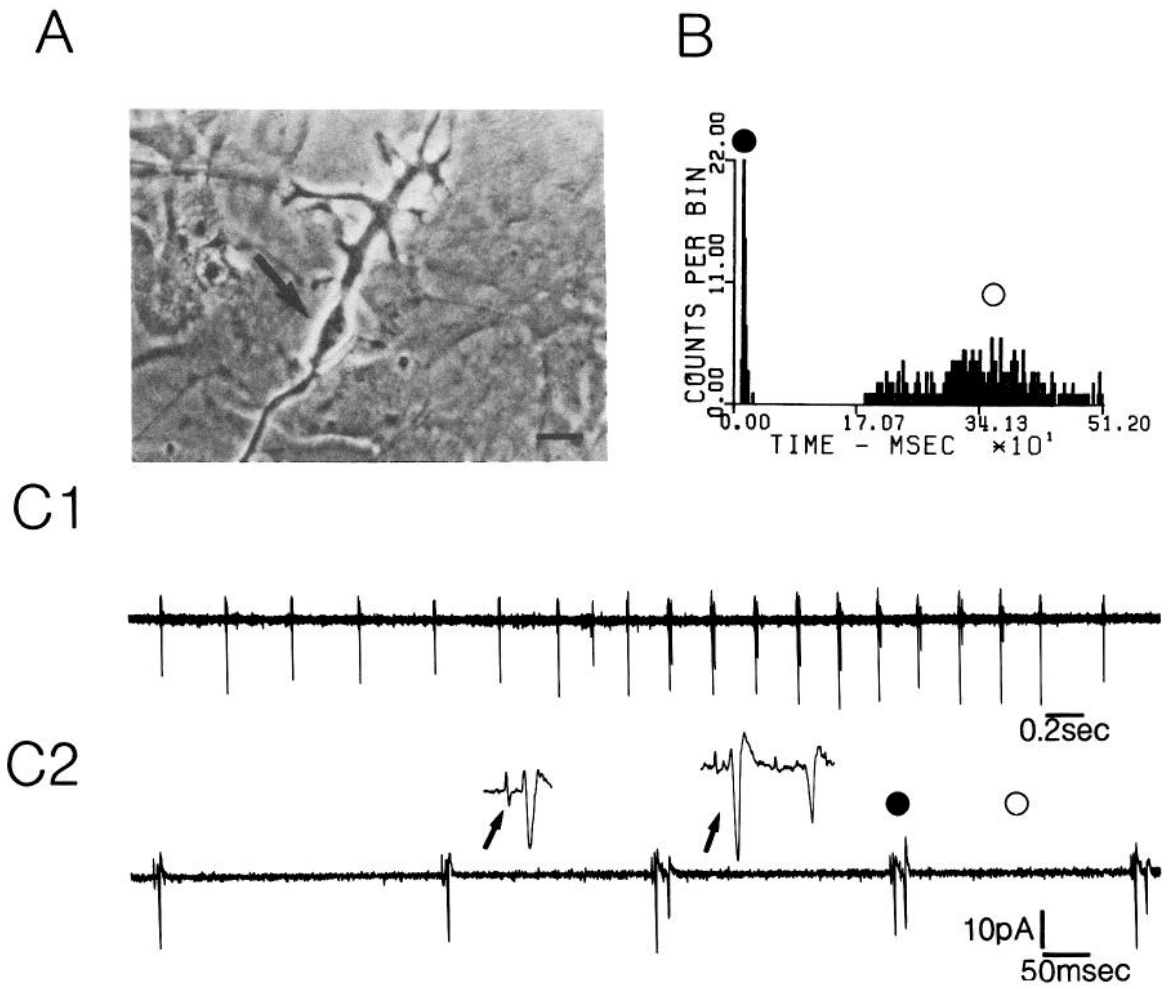


*Figure 10.* Extracellular recordings from a 16 d PN exhibiting a regular pattern of complex spikes. The PN is shown in the phase-contrast micrograph (*arrow*). This PN was classified as *somatic and dendritic maturation* in terms of its stage in morphological development. The AC-coupled recordings are shown at 2 time bases in *C1* and *C2* and the corresponding ISI-histogram in *B*. The interspike intervals marked with symbols on the recording indicate the intervals generating the similarly marked peaks in the histograms. Each event is composed of multiple spikes, with the number of spikes/event varying. In some cases, the first spike was considerably smaller and of a different waveform from the remaining spikes, suggesting that it was generated at a distant site.

that generate a complex-type spike (Fig. 15).

By 9 DIV, dendrites were prominent in many of the PNs studied, and by 13 DIV, all PNs studied had prominent dendrites. Intracellular recordings from PNs in 13–20 d cultures revealed that synaptic activity was much more extensive in these older PNs, consistent with an increase in immunohistochemical staining for synaptic sites at these ages, predominately at a dendritic location. Spontaneous complex spikes were first observed in intracellular recordings from PNs in 9 d cultures and were common for PNs in 13 d cultures. This type of spike occurred at resting membrane potential in some PNs or could be evoked by depolarizing current pulses when the membrane potential was hyperpolarized (Fig. 16). Regular patterns of simple spikes, doublets, or complex spikes were common in 13–18 d cultures and associated with pacemaker activity, indicative of an endogenous activation. All 3 types of spikes were also associated with synaptic potentials, suggesting that synaptic input could generate all 3 types of spike events. In this case, the spikes occurred in an irregular pattern. Varying membrane potential by intracellular current injection produced changes in firing pattern and spike events similar to those observed in mature PNs. These data indicate that many of the voltage-sensitive ionic mechanisms underlying the complex membrane physiology of mature PNs had been expressed and were in the final stages of development (Fig. 16).

## Discussion

The goals of the present study were to examine the physiological development of an identified vertebrate CNS neuronal type, the PN of the cerebellum, and to correlate the stages in physiological, morphological, and synaptic development. Future studies will focus on the specific ionic conductances underlying the physiological properties characterized in the present study. For technical reasons, an *in vitro* model system, modified organotypic cultures of fetal rat cerebellum, was used. The cultures are prepared from the cortical region of fetal rat cerebella and appear to contain all neuronal types present in that region. Unlike other types of organotypic culture preparations, our preparation is well suited to immunohistochemical and high-resolution electrophysiological techniques, and it provides the visibility and accessibility necessary for a direct correlation of morphological structure and physiological function. Initially, immature PNs

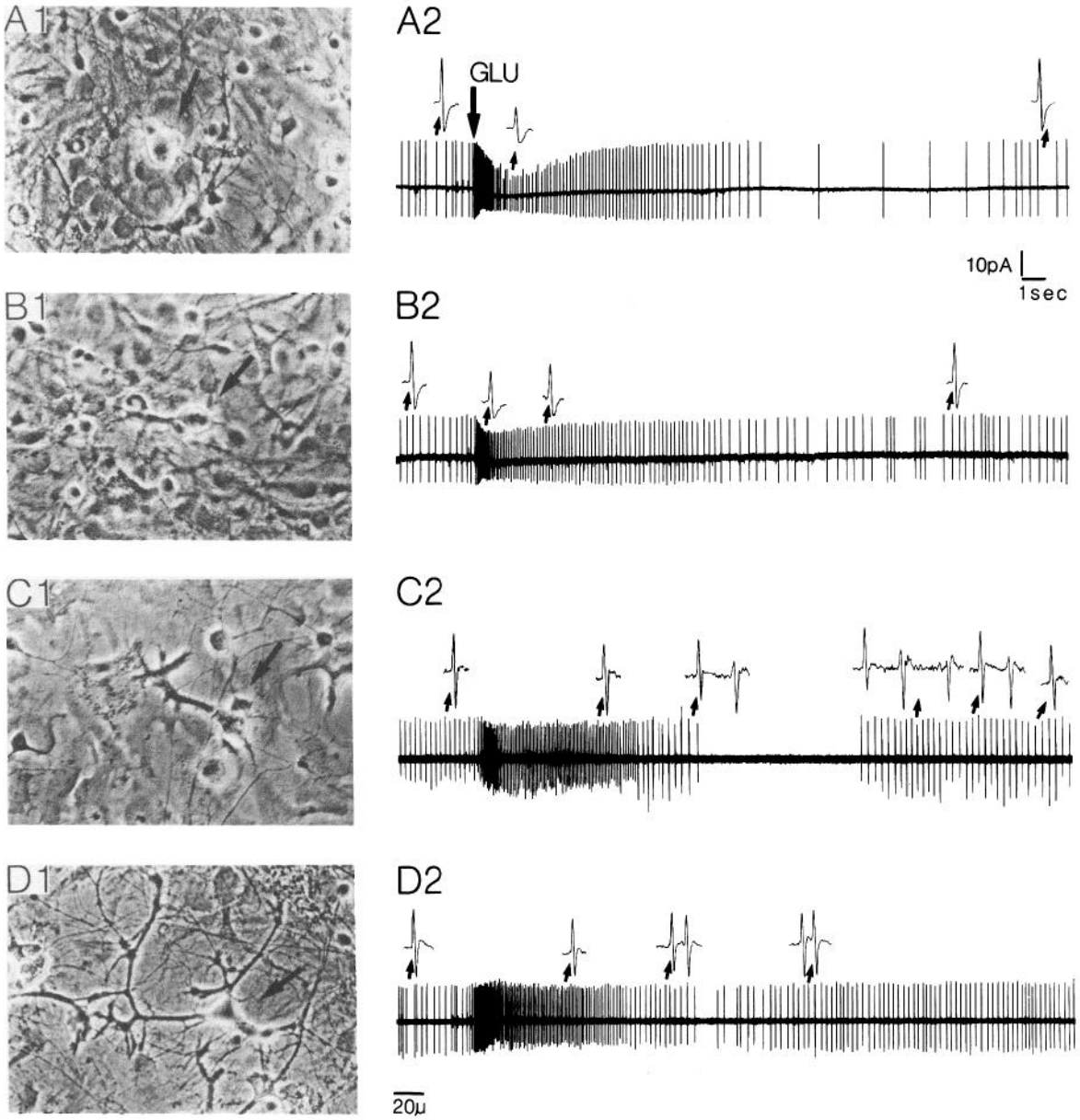


*Figure 11.* Change in the response of developing PNs to exogenously applied glutamate (*GLU*). Phase-contrast micrographs of the recorded PNs are shown at *left* (*arrows*) and sample extracellular recordings (*A2, B2* are DC coupled; *C2, D2* are AC coupled) at *right*. The PN in *A* (9 DIV) is the most immature, consisting of a large soma but no apparent dendritic structure. The PNs in *B1* (9 DIV), *C1* (9 DIV), and *D1* (14 DIV) show increasing morphological maturation, including the development of a complex dendritic tree. All PNs exhibited a regular simple spike firing pattern. The response to GLU for the PNs shown in *A1* and *B1* consisted of an increase in simple spikes followed by a period of reduced activity. The increased activity was associated with a baseline shift (not evident in the AC recordings in *C2, D2*; *A2, B2* are DC recordings) and a decrease in amplitude of the spike events, suggesting a depolarization of the membrane mediated the increase in firing rate. At older ages, the response was more complex and usually included periods of doublet or burst firing, in addition to the increase in simple spike firing and periods of inhibition. These changes in the GLU response with development may be due to maturation of membrane properties such as the voltage-sensitive conductances, the development of dendritic structure and associated conductances, or a change in the conductance activated by GLU.

could not be identified in young cultures. However, immunostaining of the cultures with the cGPK antibody revealed the morphological characteristics of the immature PNs and demonstrated that at all developmental stages the PNs exhibit distinct morphological features.

The cultures are prepared from 20 d rat embryos, a day before birth. Studies of cerebellar development *in vivo* have shown that the cerebellum is relatively immature at this age, with the majority of development occurring rapidly and over a condensed period during the first 3 weeks of the postnatal period (Eccles, 1970; Altman, 1972, 1982; Altman and Bayer, 1978; Hendelman and Aggerwal, 1980). Our immunohistochemical studies have demonstrated that PNs derived from immature tissue undergo extensive morphological development in culture and that the stages in morphological development are remarkably similar to those described for PNs *in vivo*. In culture the maturation process extended for a 3 week period, similar to the time course for development *in vivo* (Woodward et al., 1969a, b; Altman, 1972, 1982).

The most obvious morphological differences between PNs

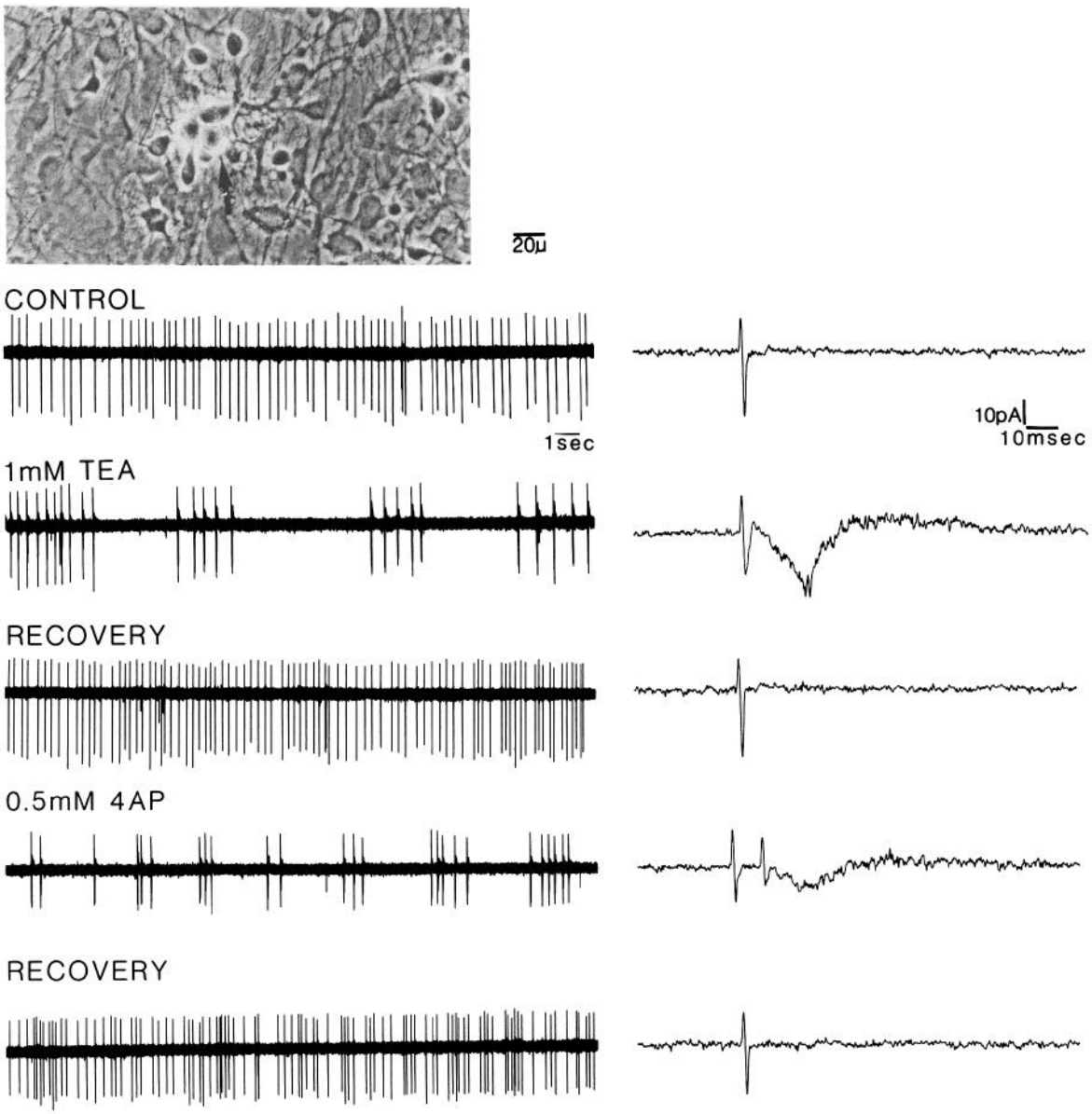


*Figure 12.* Effect of $K^+$ channel blockers on the spontaneous activity of an 8 d PN shown in the phase-contrast micrograph at the *top* (*arrow*). This PN exhibited a regular simple spike firing pattern. The *trace* at *right* shows the spike events at a faster time base. TEA, at concentrations known to block the $Ca^{2+}$-activated $K^+$ conductance, and 4-AP, at concentrations known to block the A-current, changed the simple spike pattern into intermittent patterns. The large baseline shift following the spike events is interpreted as a prolonged membrane depolarization, based on similar studies using intracellular recording techniques. With 4-AP the spike events were converted from simple spikes to burst discharges consisting of 2 or more spikes. DC recording.

differentiated in culture compared to those differentiated *in vivo* relate to the dendritic structure. The dendritic tree of PNs differentiated in culture is smaller and less developed than PNs differentiated *in vivo,* and the cultured PNs usually have several large dendrites emerging from the somatic region, instead of the one primary dendrite characteristic of PNs *in vivo.* Similar morphological differences have also been noted for mature PNs in other types of cerebellar culture preparations (Palacios-Pur et al., 1976; Calvet and Calvet, 1979; Calvet et al., 1979; Seil, 1979; Hendleman and Aggerwal, 1980; Gahwiler, 1981; Moonen et al., 1982), in the cerebella of certain mutant mice (Sidman, 1968; Sotelo, 1975), and in cerebella in which the number of interneurons or the afferent input has been experimentally reduced (Altman and Anderson, 1972; Llinás et al., 1973; Herndon and Oster-Granite, 1975; Woodward et al., 1975; Yu, 1975; Bradley and Berry, 1976; Crepel et al., 1980). These alterations in dendritic structure are thought to result from abnormal cellular migration and a reduction in the number of parallel fiber synapses, consequent to the disruption of the normal 3-dimensional organization of the cerebellum. Despite these differences in dendritic structure, mature PNs in culture exhibited many of the electrophysiological properties and activity patterns characteristic of PNs *in vivo.* This similarity may be due to the fact that ionic conductances located in the somatic region play a major role in establishing the activity patterns characteristic of the mature neurons.

Our immunohistochemical studies with an antibody to synapsin I revealed that synapse formation was initiated in culture

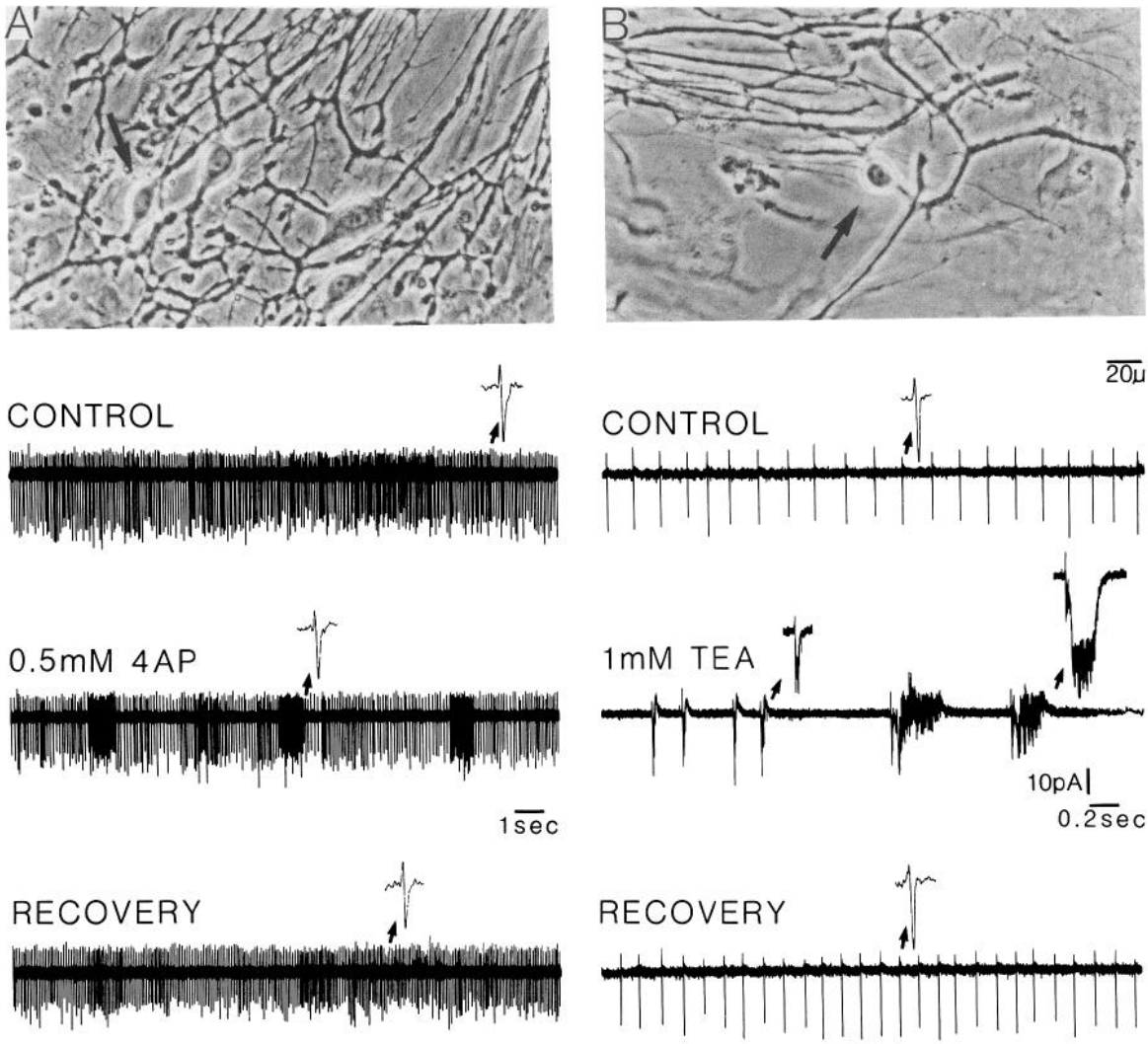


*Figure 13.* Effect of $K^+$ channel blockers on the spontaneous activity of two 17 d PNs shown in the phase-contrast micrographs at the *top* (*arrow*). Both PNs exhibited a regular simple spike firing pattern. In the PN shown in *A*, 4-AP caused variations in firing rate but did not produce the large baseline shifts observed in younger PNs. The effect of TEA was similar to that observed in younger PNs, but the baseline shifts were more pronounced. AC recordings; *inserts* for the TEA record are DC recordings showing the baseline shift associated with the spike events.

and was coupled to morphological maturation. Synapse formation was initiated at an early stage in development, when PNs were morphologically and electrophysiologically quite immature. The main period of synapse formation was concurrent with the main period of dendritic development, reflecting the fact that dendrites are the main cellular site for synaptic input in the PNs *in vivo* and in culture. Electrophysiological recordings from the immature PNs demonstrated that many of the early synapses were excitatory in nature, producing slow patterns of spontaneous activity. This input was derived from granule cells, the only excitatory neurons in the cultures. *In vivo,* initial synaptic contacts are present on the somata and perisomatic processes of the PN by 3–5 d after birth and are derived primarily from the climbing fibers, axons originating from neurons in the inferior olivary nucleus (Woodward et al., 1971; Altman, 1972). However, transient parallel fiber (axons of the granule cells) innervation is also thought to be present at this time (Woodward et al., 1971; Altman, 1972; Crepel, 1974; Shimono et al., 1976), suggesting that the early innervation of PNs by granule cells in culture is reflective of the developmental pattern *in vivo.* The timing of inhibitory synaptic input was not investigated in the present study. However, inhibitory synaptic potentials were prominent in intracellular recordings from PNs in 13–16 d cultures, indicating that inhibitory synaptic input was established by this culture age. Inhibitory input to the cultured PNs presumably arises from the basket and stellate cells, based on the known innervation *in vivo* (Eccles, 1967; Palay and Chan-Palay, 1974), and the relationship between cell types in culture. *In vivo,* numerous synaptic profiles attributed to basket cells are present on the PN soma by 12 d after birth (Woodward et al., 1971; Altman, 1972; Crepel, 1974; Shimono et al., 1976). Synaptic profiles derived from granule cells and stellate cells also appear at this time, and extensive innervation of the dendrites is established by 21 d after birth. Extensive dendritic innervation is also a characteristic of PNs differentiated in culture.

In the cultures, mature PNs exhibit both simple and complex spikes and commonly fired in regular patterns of simple spikes, reflective of activity observed *in vivo.* This similarity indicates that many of the electrical and chemical mechanisms that generate the activity of PNs *in vivo* are also expressed in the cultures

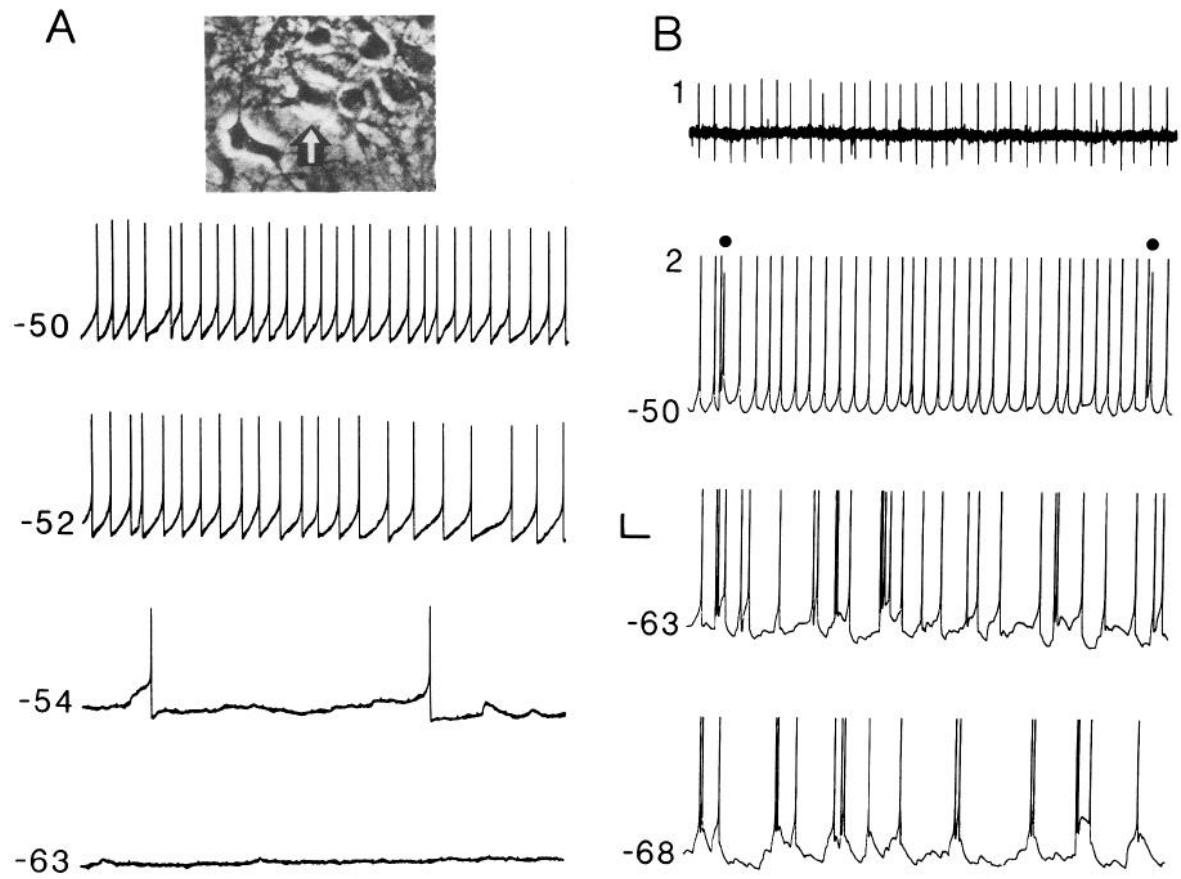


*Figure 14.* Intracellular recordings from a 6 d (*A*) and 17 d (*B*) PN exhibiting the regular simple spike firing pattern. The 6 d PN is shown in the phase-contrast micrograph in *A* (*arrow*) and was characterized as *apical cone or fusiform stage.* The 17 d PN displayed a morphology characteristic of a well-developed PN. In *B,* the initial extracellular recording is shown in *trace 1,* and the subsequent intracellular recording in *trace 2.* In both the mature and immature PN, the spontaneous activity was generated by synaptic and endogenous mechanisms. The regular, simple spike pattern, observed at membrane potentials around −50 mV, is generated by slow membrane depolarizations (pacemaker potentials) that evoke spikes. When the membrane potential is hyperpolarized, the regular pattern of activity is not observed, indicating the voltage-sensitive nature of the ionic mechanisms underlying this activity. The irregular patterns of activity revealed by membrane hyperpolarization are associated with synaptic potentials and are considered to be derived from synaptic input. The events marked with *dots* in trace 2 of *B* are thought to arise from synaptic input, based on the irregular and infrequent nature of the events. Considerably more synaptic activity is evident in the older PN (*B*), consistent with the increase in immunostaining to synapsin I, indicative of synaptic sites, in older PNs. Recordings were made with patch electrodes filled with high-$K^+$ saline. Because of the alteration in $Cl^-$ gradient caused by this electrode solution, the proportion of the synaptic potentials that is EPSPs or inverted IPSPs mediated by $Cl^-$ is not known. Calibration bar, 10 mV, 0.2 sec. Spike amplitude is not fully reproduced.

and supports the hypothesis that many aspects of cerebellar development—including PN maturation, synapse formation, and expression of appropriate electrical and chemical excitability—occurs in culture. Intracellular recordings from the cultured PNs revealed that both synaptic and endogenous mechanisms contribute to these patterns of activity and that membrane potential was an important factor in determining the type of spike event generated and regularity of the activity. The 2 main spike events generated by PNs, the simple and complex spike, were identified as all-or-none events that could be triggered by either synaptic input or the endogenous mechanisms. Some neurons appear to possess only the mechanisms to generate one type of spike event (single or complex spike), whereas other PNs possess the mechanisms to generate both. Regularly occurring patterns of activity were intrinsically generated by voltage-sensitive mechanisms; the irregular patterns were initiated by synaptic events.

*In vivo* simple spike activity can be generated in the PN by parallel fiber input, but an endogenous component to simple spike activity has been implicated by several types of studies in which the input from granule cells, the majority source of the excitatory input to the PNs, was altered (Snider et al., 1967; Gahwiler et al., 1973; Calvet et al., 1974; Woodward et al., 1974; Calvet and Lepault, 1975; Siggins et al., 1976; Seil, 1979). For example, removal of the extracerebellar input or reduction of granule cell population does not significantly alter the firing rate of PNs, a change that would be expected if the activity was strongly dependent on excitatory input (Snider et al., 1967; Woodward et al., 1974; Siggins et al., 1976). Thus, it appears that the endogenous mechanisms also play a prominent role in defining the simple spike firing pattern of PNs *in vivo.*

*In vivo* complex spikes are initiated by synaptic input from the climbing fibers and are associated with a large membrane depolarization and a progressive spike inactivation (Granit and Phillips, 1956; Eccles et al., 1967; Murphy and Sabah, 1971; Crepel and Delhaye-Bouchaud, 1978; Ekerot and Oscarsson, 1980; Llinás, 1981; Ito, 1984). Early theories attributed the large membrane depolarization to a strong, simultaneous input from climbing fiber branches (Eccles, 1967). However, the all-or-none nature of the response suggests that a critical component may be contributed by additional, nonsynaptic ionic mechanisms

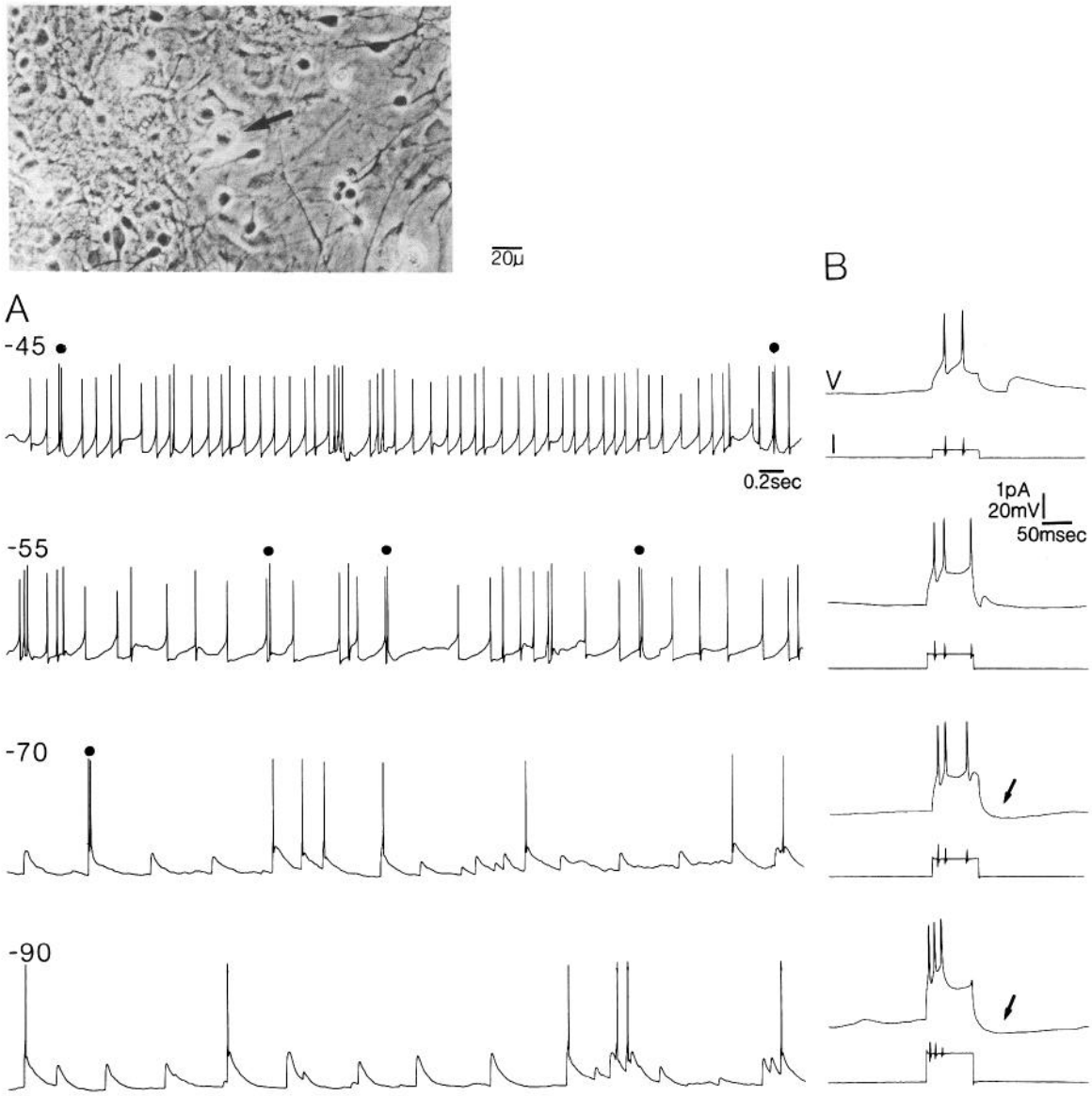


*Figure 15.* Intracellular recording from a 7 d PN exhibiting spontaneous activity consisting of regular simple spikes and doublets (*dots*). The PN, shown in the phase-contrast micrograph at the *top* (*arrow*), was characterized as *apical cone or fusiform stage* in terms of its morphological development. When the membrane potential was hyperpolarized by intracellular current injection, the frequency of the activity varied in a membrane potential-dependent manner. Synaptic events were observed at the hyperpolarized membrane potential and occurred at a frequency comparable to the frequency of doublet spikes. These data suggest that endogenous mechanisms generated the basic regular simple spike pattern, with the doublet events superimposed on the basic pattern resulting from synaptic input. In *B* is shown the voltage response produced by depolarizing current pulses at each membrane potential. When the membrane potential was hyperpolarized, the response evoked by depolarizing current pulses changed from a repetitive simple spike pattern to a burst discharge, which may be an early form of the complex spike. *V,* voltage trace; *I,* current trace. Recording made with a patch electrode.

intrinsic to the membrane. This possibility is supported by recent *in vivo* studies showing that complex spikes similar to those evoked by climbing fiber input can be generated by other mechanisms (e.g., current pulses, granule cell input in the absence of inhibitory input; Campbell et al., 1983a, b). The fact that the cultured PNs, which do not receive climbing fiber input, can generate complex spikes either spontaneously or in response to current pulses, also supports a role for intrinsic membrane mechanisms in the generation of this response.

Although the electrophysiological properties of mature PNs have been examined in some detail, there have been relatively few studies on the electrophysiological properties of immature PNs or the time course and sequence in the expression of the mature properties. The majority of information comes from *in vivo* studies, in which extracellular recording techniques were used. There appears to be general agreement among the *in vivo* studies that sensitivity to transmitters is expressed at an early stage of PN development, that immature PNs exhibit spontaneous activity, and that the frequency and pattern of PN activity changes during the postnatal period (Woodward et al., 1969a, b, 1971; Crepel, 1972). However, little is known about the cellular, membrane or ionic basis for the activity, or the changes in activity associated with the maturation process.

*In vivo* spontaneous activity can be recorded from PN around 1–3 d after birth and consists of slow doublet spikes. Around 1 week after birth this pattern evolves into a slow, regular pattern

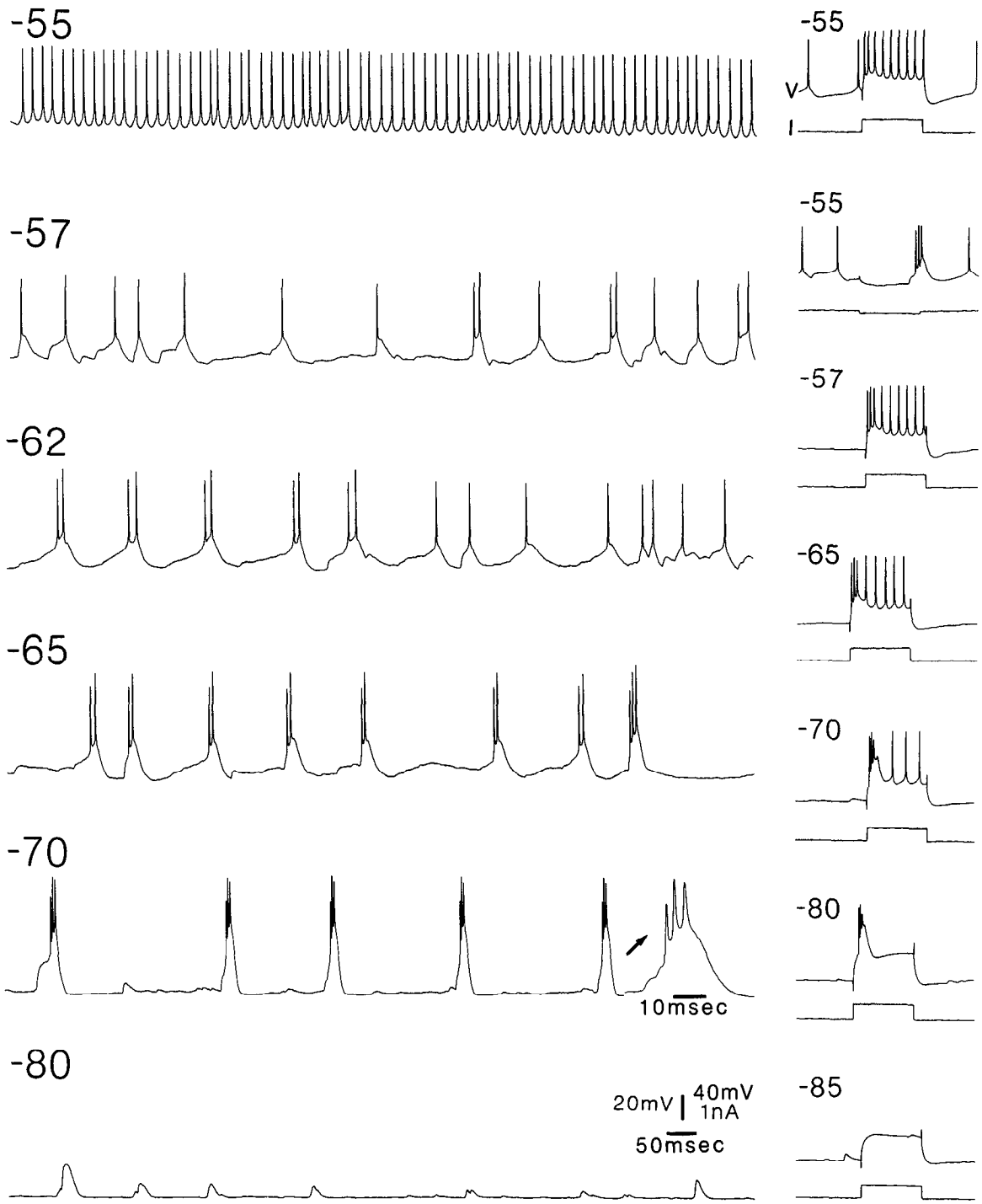


*Figure 16.* Intracellular recording from a 17 d PN. The PN exhibited a regular simple spike firing pattern. The firing rate and the type of spike events were altered by hyperpolarizing the membrane potential in a manner similar to that observed in mature PNs. Voltage responses evoked by depolarizing current pulses at each membrane potential consisted of an initial burst discharge followed by repetitive simple spikes (except for membrane potentials of −80 and −85 mV). These data suggest that many of the complex membrane properties exhibited by mature PNs have been expressed by this stage of development, although they may not be totally mature. Conventional intracellular recording techniques used. $K^+$-acetate-filled electrodes.

of simple spikes. A complex spike pattern also appears at this time, superimposed on the simple spike pattern. The regular simple spike pattern continues up to day 9 and then rapidly evolves into adult patterns by 3 weeks after birth. These transitions in firing pattern appear to correlate with synaptic development (Woodward et al., 1969a; Altman, 1972; Crepel, 1974; Shimono et al., 1976), suggesting that synaptic components may play a major role in defining the firing patterns of immature PNs. However, a contribution of inherent pacemaker mechanisms has also been proposed to account for the sustained periods of regular activity (Woodward et al., 1969a). In these *in vivo* studies, a direct correlation between type of PN activity

and synaptic or morphological development was not possible, because PN development *in vivo* is not tightly coupled to postnatal age (i.e., a variety of developmental stages are expressed at any particular postnatal age), and the PNs examined with electrophysiological techniques were not individually visualized.

Extracellular recordings from immature PNs in 4–20 d cultures revealed that the electrophysiological properties of PNs differentiated in culture also change during the developmental period and that transitions observed in culture generally reflected those observed *in vivo*. In culture, the electrophysiological properties of the developing PNs could be directly correlated with morphological development and intracellular recordings used to identify the membrane events underlying the patterns of activity and changes associated with the developmental process. These studies demonstrated that expression of electrophysiological properties is initiated by 4 DIV in the cultured PNs, the earliest culture age studied. At this age, the majority of PNs were very immature both morphologically and electrophysiologically. However, the chemical and ionic mechanisms associated with sensitivity to the transmitter GLU, and the ionic mechanisms responsible for action potential generation had already developed in many of the immature PNs. From this starting point, 3 transitions in the expression of mature properties were identified; the first transition occurs between 4 and 6 DIV, when the capability for generating spontaneous activity develops; the second transition occurs between 6 and 9 DIV, when early patterns of spontaneous activity are converted to regular patterns of simple spikes; the third transition occurs between 9 and 13 DIV, when complex spikes are expressed.

Relatively few of the PNs in 4 d cultures exhibited spontaneous activity, and these PNs exhibited slow, irregular patterns of simple spikes. Intracellular recordings demonstrated that this pattern was synaptically generated. Early doublet patterns reflective of those reported for PNs *in vivo* were not observed in 4 d cultures but were observed at older culture ages. By 6–8 d in culture, which is still before the main period of synaptic and dendritic maturation, the majority of PNs were spontaneously active, exhibiting regular, intermittent or irregular patterns of activity consisting of simple spikes or simple spikes and doublets. These patterns and spike events resembled activity reported for PNs of similar age *in vivo*. Intracellular recordings from the cultured PNs demonstrated that these patterns are generated by synaptic input and endogenous, voltage-sensitive mechanisms, the endogenous mechanisms producing regular patterns of activity. As for mature PNs, several types of ionic mechanisms were required for expression of the endogenously generated activity, including $Na^+$, $Ca^{2+}$, and at least 2 types of $K^+$ channels. However, in the immature PNs, the endogenous mechanisms produced a significantly lower firing rate than in mature PNs, suggesting that either further maturation of the underlying ionic mechanisms or additional ionic components were required for full expression of the mature pattern. In immature PNs that did not exhibit endogenously generated activity, depolarization of the membrane by current pulses revealed that these PNs had already developed the capability for repetitive firing. Thus, it appears that during PN maturation expression of the mechanisms underlying repetitive firing precedes expression of the mechanisms responsible for sustaining the activity (pacemaker activity). Mechanisms responsible for complex spike generation were not evident at the early developmental stages, but were associated with older PNs exhibiting dendritic structure. Thus, expression of ionic mechanisms underlying this type of spike appears to be a feature of later developmental stages.

The expression of endogenously generated activity in the PN occurs at a stage of development when dendrites are absent or rudimentary and does not correlate with the extent of dendritic development. Thus, it is likely that the ionic mechanisms mediating this activity are located in the somatic region. The more complex properties, including the mechanisms that generate the complex spike, are expressed later in development, when the somatic and dendritic regions are fairly well developed and synapse formation is extensive, suggesting a predominately dendritic location. A location of the ionic mechanisms mediating the complex spike in the dendritic region is an attractive possibility and consistent with a role in the climbing fiber response (Eccles et al., 1967; Llinás, 1981). The early expression of endogenously generated electrical activity in the PN raises the possibility of a functional role in the maturation process. One such role may be in regulating $Ca^{2+}$ influx and, as a consequence, intracellular $Ca^{2+}$ levels. $Ca^{2+}$ is known to have multiple effects on cellular metabolism and excitability (Llinás and Walton, 1980; Kretsinger, 1981) and has been postulated to regulate growth and development (Llinás and Sugimori, 1979).

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