INTRODUCTION

Cerebellar nuclei (CN) play a crucial role in cerebellar circuitry. They provide the sole output signals from the cerebellum, which are sent to various structures in the brain stem, midbrain, and thalamus and, by way of the latter also reach the cerebral cortex. The CN receive input from three major sources: the excitatory mossy and climbing fibers that project to the cerebellar cortex and provide collateral connections to the CN (Eller and Chan-Palay 1976; Kitai et al. 1977; Mihailoff 1993; Shinoda et al. 1993) and the inhibitory Purkinje cell axons (Ito et al. 1970), which contribute the majority of synapses contacting CN neurons (Chan-Palay 1977; Teune et al. 1998).

It has been established that the CN, based on their morphology, their projection targets, and the transmitters used, consist of at least three types of neurons (Chan-Palay 1977; De Zeeuw et al. 1989; Schwarz and Schmitz 1997; Teune et al. 1998). Two types are projection neurons while the third type includes the smallest cells which are assumed to confine their axonal reach to the CN (Chan-Palay 1977). Glutamate (Schwarz and Schmitz 1997; Verveer et al. 1997) and GABA (De Zeeuw et al. 1989) are used as transmitters and divide projection neurons into excitatory and inhibitory types, respectively. Glutamatergic projection neurons carry most of the output from the cerebellum and send their axons to several brainstem and midbrain structures as well as to precerebellar structures such as the pontine nuclei (Angaut et al. 1985; Faull 1978; Faull and Carman 1978; Schwarz and Schmitz 1997). These neurons display the biggest somata and dendritic trees within the CN cells. The smaller GABAergic projection neurons carry feedback signals to the inferior olive and are not known to project elsewhere (De Zeeuw et al. 1989). There is evidence that both types of projection neurons receive GABAergic input from Purkinje cells (De Zeeuw and Berrebi 1995; Teune et al. 1998). The interneurons, whose input and output connections are unknown, are believed to colocalize both GABA and glycine as transmitters (Chen and Hillman 1993). An unresolved issue is whether the different classes of neurons possess different membrane properties. Studies of CN neurons in brain slices (Aizenman and Linden 1999; Gardette et al. 1985; Jahnsen 1986a,b) and in a hindbrain preparation (Llinás and Mühlethaler 1988b) noted some variability in the electrogenic membrane responses of different CN cells. No attempt was made, however, to differentiate functional groups of cells based on their intracellular properties or to relate them to anatomical or pharmacological features. The classes of CN neurons described earlier in this paragraph are known to differ in soma size. Because previous studies used high-resistance microelectrodes, which are probably biased to preferentially penetrate neurons with large somata, it is questionable if neurons from all classes could be recorded. We therefore resorted to the whole-cell patch method to record from CN cells to address the questions raised. Using this method, somata of CN cells were visualized using the infrared Nomarski interference technique so that...
recordings could be made from even the smallest cells within the CN. We report here that a subset of cells with somata and dendritic fields in the smallest range show membrane properties that are clearly distinct from the majority of cells. Preliminary results were published in abstract form (Czubayko et al. 1998).

METHODS

Sprague-Dawley rats (12–21 days old) were deeply anesthetized with ketamine; this was followed by transcardial perfusion with ice-cold modified artificial cerebrospinal fluid (ACSF) in which NaCl had been replaced by sucrose. The modified ACSF contained (in mM) 125 sucrose, 2.5 KCl, 1.25 NaH2PO4, 3 MgCl2, 26 NaHCO3, 0.1 CaCl2, and 20 d-glucose and was oxygenated with 95% O2-5% CO2. Brains were removed carefully from skulls and put in ice-cold modified ACSF. The block of tissue containing the CN was isolated by two frontal cuts, one at the level of the colliculus and the other just caudal to the cerebellum. The brain stem was removed by a horizontal cut along the fourth ventricle. The hemispheres were separated by a sagittal cut and each hemisphere was glued, with the vermis side down, to a metal block. Parasagittal slices (300 µm) of the CN were prepared using a vibrating microtome (Leica, Bensheim, Germany). The slices were stored in modified ACSF at room temperature for 30 min before the liquid was replaced with normal ACSF containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 2 MgCl2, 26 NaHCO3, 2 CaCl2, and 20 d-glucose and oxygenated with 95% O2-5% CO2. The slices were kept for 1 h more before they were transferred to a submerged recording chamber. During recordings, the slices were continuously superfused with ACSF at room temperature. Drugs were diluted in ACSF to their final concentration and were saturated with 95% O2-5% CO2 before use. The slices were superfused with the solution containing the drug for at least 10 min before the steady state effects were recorded. All drugs were obtained from Sigma (St. Louis, MO). To avoid precipitation, no phosphate ions were added to ACSF containing cobalt. Cobalt and cesium were used in a concentration of 2 mM.

Patch clamp intracellular recordings were performed with glass microelectrodes that had a resistance of 5–20 MΩ when filled with an internal solution containing 131 mM K-gluconate, 5 mM NaCl, 5 mM K+ HEPES, 5 mM EGTA, 4 mM K+ ATP, 0.3 mM Na+ guanosine 5’-triphosphate, 0.5 mM CaCl2, and 4 mg/ml neurobiotin and adjusted to pH 7.3 with KOH. For recording the membrane potential, an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) in the current clamp-bridge mode was used. The voltage records were low-pass filtered (cutoff frequency 10 kHz) and digitized at sampling rates of 5–20 kHz with a personal computer equipped with a 1401plus interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK). Spike2 and Matlab software (Mathworks, Natick, MA) was used to analyze data. Statistical testing was performed with Statistica (StatSoft, Tulsa, OK).

Action potential amplitude and width were measured during spontaneous activity for a period of time in which no bias current was injected. Amplitude was defined as the difference of action potential peak and the minimum of the afterhyperpolarization (AHP) following the spike. Spike width was measured as time at half-amplitude. The firing threshold was determined similarly to the method used by Azouz and Gray (1999). We calculated the first derivative of the voltage recordings and computed the standard deviation (SD) of the noise over a period of time when no action potential was generated. We then took the voltage at the point in time when the derivative reached a value of 5 × SD as an estimate of the firing threshold. All results obtained with this analysis were checked for plausibility by visual inspection of the traces of the action potential at a small time scale.

Comparisons of voltage responses with current stimulation between neurons were done after a common “stimulus strength” was selected. Stimulus strength was defined as the product of input resistance and current amplitude. It corresponds to voltage deflection, which would have been obtained if the intracellular current pulse had not activated any voltage-dependent conductances other than those active at the potential before stimulus onset (prestimulus potential). The stimulus strength for current pulses in the present study was between 10 and 20 pA × ms.

For extracellular recordings of single-unit activity, tungsten microelectrodes were positioned in the nucleus lateralis. The signal was band-pass filtered and digitized at a sampling rate of 10 kHz. To investigate periodicity in spike trains, autocorrelation functions were calculated. A threshold discriminator was applied to extract spike times from voltage traces recorded intracellularly or extracellularly. For each spike train, the autocorrelogram and its respective 0.99 confidence interval were computed for a window of −2.5 to 2.5 s at a bin width of 1 ms (Abeles 1982). The autocorrelogram was then smoothed with a digital Gaussian filter (width, 3 bins). In autocorrelograms showing rhythmicity, non-central peaks on one side of the zero, which exceeded the upper 0.99 confidence interval, were counted as being significant.

Statistical testing of differences in the parameters of both types of neurons was performed by computing a one-way multivariate variance analysis (one-way MANOVA). All parameters were tested for normality (Kolmogorov-Smirnov test). Post-hoc analysis to locate the parameters contributing to the difference between the populations of cells was done with Scheffé tests for normally distributed variables (Scheffé 1959) and the Mann-Whitney test for variables not distributed normally.

During recording, cells were filled with 0.4% neurobiotin for 0.5–3 h. At the end of the recording, the pipette was withdrawn carefully to avoid damage to the cell. Although this was routinely possible for large cells, it turned out to be much more difficult for small cells with presumptive small dendritic fields. Some of the small cells were pulled out of the slice by the forces of the seal. Furthermore, the nuclei of the small cells tended to be sucked out by the pipette during withdrawal, which resulted in damage and insufficient staining of the cells. Thus the rate of successful fillings of small cells was much lower as compared with cells with large somata and/or large dendritic fields. After withdrawal of the pipette, the slices were immediately immersion-fixed overnight in a phosphate-buffered solution containing 4% paraformaldehyde (0.1 M, pH 7.4, 4°C). To avoid nonspecific staining, slices were treated with 3% H2O2 to block endogenous peroxidase. Slices were then incubated with an avidin–biotin complex peroxidase. Slices were then incubated with an avidin–biotin complex (1:100) and(Vector Laboratories, Burlingame, CA) for 1 h. After the incubation, the slices were washed for 3 h. At the end of the staining, the pipette was withdrawn carefully. Well-filled neurons were drawn using a camera lucida at a final magnification of 900x.

RESULTS

Definition of two types of neurons in the CN

Our sample comprises 70 CN neurons recorded in whole-cell patch current clamp mode. All cells were recorded in slices obtained from animals 12–21 days old. It has been established that intrinsic and synaptic membrane conductances mature during the first postnatal week (Gardette et al. 1985). Our recordings confirm and extend this earlier finding because we did not detect any change in basic membrane properties in the age range investigated (see Table 1). Furthermore, the basic membrane properties found by the present study are generally comparable to the ones found in slices taken from adult guinea pigs, which indicates that the membrane properties of rats at postnatal day 12 have reached an adult-like pattern (Jahnsen 1986a; Llinás and Mühlethaler 1988b). Cells were included in
TWO TYPES OF NEURONS IN THE CEREBELLAR NUCLEI

Table 1. Classification of CN neurons: summary of the electrophysiological parameters of two types of CN neurons

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Type I</th>
<th>Type II</th>
<th>Scheffé: P</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>63</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>15.6 ± 2.5</td>
<td>15.1 ± 2.0</td>
<td>0.62/0.55</td>
<td>63/7</td>
</tr>
<tr>
<td>Input resistance, GΩ</td>
<td>0.61 ± 0.51</td>
<td>1.25 ± 0.55</td>
<td>0.04/0.007*</td>
<td>54/7</td>
</tr>
<tr>
<td>Time constant, ms</td>
<td>74.6 ± 32.8</td>
<td>69.3 ± 36.5</td>
<td>0.61/0.51</td>
<td>54/7</td>
</tr>
<tr>
<td>Spike width, ms</td>
<td>1.44 ± 0.42</td>
<td>1.86 ± 0.46</td>
<td>0.04/0.01</td>
<td>52/7</td>
</tr>
<tr>
<td>Slope, V/s</td>
<td>209 ± 72</td>
<td>200 ± 93</td>
<td>0.78/0.72</td>
<td></td>
</tr>
<tr>
<td>Firing threshold, mV</td>
<td>−38.8 ± 12.7</td>
<td>−38.4 ± 6.2</td>
<td>0.44/0.31</td>
<td>52/7</td>
</tr>
<tr>
<td>Spike amplitude, mV</td>
<td>66.4 ± 10.41</td>
<td>78.02 ± 8.64</td>
<td>0.06/0.02</td>
<td>52/7</td>
</tr>
<tr>
<td>Plateau discharge</td>
<td>22.57 ± 20.15</td>
<td>0</td>
<td>0.049/0.01*</td>
<td>49/7</td>
</tr>
<tr>
<td>Difference firing threshold – ADP, mV</td>
<td>7.46 ± 3.6</td>
<td>16.98 ± 4.1</td>
<td>0.0002/0.001</td>
<td>52/7</td>
</tr>
<tr>
<td>Average firing rate at rest, spikes/s</td>
<td>13.6 ± 6.0</td>
<td>7.2 ± 2.9</td>
<td>0.06/0.02</td>
<td>52/7</td>
</tr>
<tr>
<td>Low/high threshold calcium potentials</td>
<td>+</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium plateau</td>
<td>+</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delayed inward rectification</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The parameters in the rows 2–11 (age to average firing rate at rest) were taken to compute a one-way multivariate variance analysis (MANOVA). Significance levels for each parameter as yielded by Scheffé post-hoc comparison are given for normally distributed variables. For variables not normally distributed (+), the non-parametric Mann-Whitney test was applied. ADP, afterdepolarization. One-way MANOVA, P = 0.000031.

Intracellular properties that we will refer to as type I (bursting neurons) and type II (non-bursting neurons).

Characteristics that allowed us to differentiate type I and II neurons...
neurons can be divided into three categories. The first category encompasses various membrane properties that are functionally related to the presence or absence of burst firing. Among these properties are potentials related to the generation of plateau potentials, rebound activity, and a slow afterhyperpolarization after a long spike train or after a burst (burst-AHP). The second category comprises the form of the action potential and its afterpolarizations, which were found to be different in the two types of neurons. Among these parameters are action potential width and amplitude and the sequence of afterpolarizations. The third category encompasses the morphology of the two types of neurons as revealed by intracellular fillings and observation by infrared video microscopy.

**Plateaus, rebound potentials, and burst-AHPs**

Type I and II neurons were found to differ distinctly in the generation of plateau potentials in response to intracellular current injection. Whereas type I neurons generated long plateau potentials in response both to depolarizing and to hyperpolarizing current pulses, type II neurons did not (Fig. 1, C–F). After short suprathreshold depolarizing current pulses (300 ms), type I neurons typically generated plateau potentials topped with high-frequency trains of action potentials (Fig. 1C). Such bursts resembled the ones seen with negative bias currents. Suprathreshold current amplitudes always generated full bursts in an all-or-none manner. A single action potential could never be evoked. As a quantitative estimate of plateau generation following short depolarizing pulses, we measured the excess length of the plateaus after the pulse by taking the time from the end of the pulse to the point of time when the membrane potential reached the prestimulus level again. Furthermore, we counted the number of action potentials generated after pulse offset. To assure comparability between different cells, those sweeps were selected that showed similar prestimulus potentials (−55 to −65 mV) and stimulus strength (see METHODS). Type I neurons displayed an excess length of 2.41 ± 2.42 s (n = 49). The number of action potentials generated during the first 700 ms after current offset was 22.5 ± 20.15. All type II neurons, in contrast, returned to the prestimulus potential immediately after the current pulse (Fig. 1D). The interval to return to the prestimulus level after pulse offset was 0.31 ± 0.32 s (n = 7) and in no case were action potentials generated after pulse offset.

During hyperpolarizing pulses, both types of neurons showed a delayed inward rectification characterized by a prominent “sag” in the voltage response after pulse onset. At pulse offset, type I neurons typically generated an all-or-none burst. In all trials, this burst resembled those that occurred with negative bias current and those that were evoked by depolarized current pulses (Fig. 1F; compare with Fig. 1, A2 and C). In contrast, type II neurons showed a smaller, graded rebound response that stayed subthreshold with small intracellular currents and that elicited only a few action potentials with higher current (Fig. 1F). We investigated the pharmacological properties of the delayed rectifier and rebound responses in both types of neurons (Fig. 2). In type I neurons, the rebound responses were studied under TTX (1 μM) to suppress sodium-dependent action potentials and plateau potentials. The lack of long-lasting rebound plateaus accompanied by high-frequency discharge of action potentials made this pretreatment dispensable for type II neurons. Rebound potentials in type I neurons had an all-or-none appearance and, in most cases, consisted of a long-lasting plateau (Fig. 2A1). In a minority of cells, the rebound response consisted of a low-threshold spike only (B1). Blockade with cobalt demonstrates that calcium channels are its basis (B2). Note the remaining graded rebound potential, which is shown to be sensitive to cesium (A4). A representative type II neuron showing delayed rectification and small rebound response (C1). Both are entirely blocked by cesium (C2).
Two types of neurons in the cerebellar nuclei

FIG. 3. Dynamics of firing rate during long suprathreshold current pulses (5 s). A: 3 type I neurons that are representative of three variants of responses found within the sample. Above the voltage traces, instantaneous firing rates during pulses are shown. A1: the most common variant of type I neuron (●) discharges a short, high-frequency spike train followed by a strong and fast adaptation in firing rate. The second variant (A2, ■) shows a long-lasting acceleration that reaches maximal firing rate after a few seconds and is followed by a slow deceleration. The third variant (A3, ○) shows a mixture of the two response patterns described in A1 and A2. In contrast with type I, a typical type II neuron (A4, □) shows a minor firing rate adaptation at the beginning of the action potential train. B: relationship between instantaneous firing rate and action potential amplitude for the 4 trains of action potential shown in A. The sequence of action potentials is plotted as a trajectory in the 2D parameter space (instantaneous firing rate and action potential amplitude; symbols and arrows mark the beginning of each train of action potentials). The trajectories of all type I neurons show large dynamics whereas the trajectory of the type II neuron shows comparatively small changes. All current amplitudes were chosen so that the same stimulus strength would be applied.

With application of long depolarizing intracellular current pulses (5 s), long trains of action potentials could be evoked and compared in both types of neurons. The dynamics of instantaneous firing rate in the action potential trains generated by the two types of neurons differed distinctly (Fig. 3; the sweeps shown are matched for stimulus strength). Type I neurons showed a larger change of instantaneous frequency during the pulse. The patterns shown by these neurons can be divided into two major variants. One variant (seen in 32 of 51 type I neurons) consisted of a high-frequency action potential discharge (≈220 action potentials/s) followed by a strong and fast adaptation in firing rate shortly after the onset of the current pulse (Fig. 3A1). The second variant (seen in 10 of 51 type I neurons) was characterized by a long-lasting acceleration that reached maximal firing rate after a few seconds, followed by smooth deceleration (Fig. 3A2). In some type I neurons, a mixture of these two patterns was observed (Fig. 3A3, n = 9). These same types of firing patterns could be observed during spontaneous burst firing. In contrast to type I neurons, the instantaneous firing rate of type II neurons during 5-s depolarizing current pulses was less dynamic and its pattern was less variable from cell to cell. In all cases, a moderate firing rate adaptation was detected at the beginning of the pulse (Fig. 3A4). Changes in firing rate in both types of neurons were always accompanied by changes in action potential parameters such as action potential width, peak of action potential, and peak of afterhyperpolarization (AHP minimum). The changes of these parameters during the current pulse were large for type I neurons and small for type II neurons. During the acceleration–deceleration phase in type I neurons, the action potentials that were generated at the highest frequencies showed the lowest peak while action potential width and AHP minimum were highest. Type II neurons typically showed somewhat higher-amplitude action potentials, more-negative AHP minima, and wider action potentials at the beginning of the pulse when their discharge rates were greatest. Figure 3B exemplifies such a relationship for the three type I neurons and the type II neuron shown in Fig. 3A by plotting the trajectory given by action potential amplitude and instantaneous frequency for the sequence of evoked action potentials after pulse onset. Trajectories for type I spike trains (n = 51) (Fig. 3A, filled symbols) all cover a large area, which indicates high changes in firing frequency and spike amplitude during the pulse, whereas the trajectory of the type II spike train (n = 7) (Fig. 3A, open square) shows almost no change in those parameters.

Population data for the time course of firing rate adaptation evoked by a depolarizing current pulse are shown in Fig. 4. Action potentials of all neurons during sweeps with the same stimulus strength were analyzed. Each action potential was plotted at the time it occurred after the onset of the pulse and the voltage and instantaneous firing rate were measured. The sweep shown is representative of the population studied, where the instantaneous firing rate was kept constant during the 5-s current pulse (n = 51). From the population data, it appears that none of the type I neurons showed significant response changes during the current pulse, whereas type II neurons did show a slight trend toward decreasing response amplitude. However, in some cases, type II neurons showed increased response amplitudes during the current pulse, which might be due to changes in the stimulus strength.
at its relative instantaneous frequency, as compared with the maximum, during the respective sweep. The mean normalized instantaneous firing rates (symbols) and their standard deviations (gray areas), calculated for bins of 100-ms width, are shown. For clarity, the comparison of the population responses of type I and type II (n = 7) neurons is shown separately for the group of type I spike trains, which showed a slow acceleration–deceleration firing pattern (Fig. 4A, n = 19), and for the remainder of the cells, which did not (Fig. 4B, n = 32). Both variants of spiking patterns of type I neurons clearly deviate from the frequency adaptation of type II neurons. The blockade of fast sodium channels by TTX unmasked potentials that are responsible for the variability of the spike pattern of type I neurons. The strong firing rate adaptation of type I neurons (Fig. 3A1) was revealed to be based on a low-threshold action potential (Fig. 4B2, n = 9). On the other hand, a slow acceleration–deceleration pattern was converted to long-lasting plateaus evoked at high potentials after the blockade of sodium channels (Fig. 4B1, n = 2). Both potentials were blocked by cobalt, which indicates that they were generated by calcium influx (not shown). The application of TTX and cobalt also reduced the probability of eliciting a spontaneous burst. Only the application of both channel blockers completely abolished the generation of spontaneous burst (not shown).

In the next step, we tested if the weak adaptation of firing rates seen in type II neurons could be converted either to the strong adaptation or to the slow acceleration–deceleration pattern seen in type I neurons. This was done by varying the prestimulus potentials and applying different current amplitudes. As shown by a representative type I neuron (Fig. 5A), the high instantaneous frequency followed by a strong adaptation at pulse onset clearly depends on the prestimulus membrane potential. At a prestimulus membrane potential of −50 mV, no initial high-frequency firing could be detected whereas it was visible in an all-or-none fashion after the membrane potential was hyperpolarized to a range between −60 and −75 mV. Notably, the level of instantaneous frequency, adjusted after the initial strong adaptation of firing, was higher in cases with lower prestimulus potentials. Comparative levels of prestimulus potential in type I cells never evoked transient high-frequency firing, which indicates that this difference in firing pattern could not be caused by the prestimulus potential (Fig. 5B). Furthermore, in contrast to type I neurons, type II neurons always showed a lower steady state firing rate for lower prestimulus potentials. In a second approach, the prestimulus potential was fixed and the current amplitude was varied (Fig. 5, C and D). This was done to explore the variability of the slow acceleration–deceleration pattern. Upon an increase in the current amplitude in a representative type I neuron (Fig. 4A and B)}
the burst-AHP rather than abolishing it (Fig. 6A2, n = 10). The slowing of the burst-AHP under cesium is consistent with the view that the waveform of the burst-AHP in type I neurons is based both on the depolarizing effect of $I_{HT}$ and on the hyperpolarizing effect of a presumptive potassium-based current. The fact that cobalt blocks the burst-AHP to a large degree suggests that this potassium current is calcium-dependent (Fig. 6A3). One possible candidate for the generation of burst-AHPs is a calcium-dependent potassium channel with small conductance (SK channel) (Blatz and Magleby 1986). However, treatment with apamin (0.2 μM), a blocker of one type of SK channel, did not abolish burst-AHPs in type I neurons (not shown; note, however, the effect of apamin on the afterpotentials following each action potential, described in the next paragraph). Type II neurons (n = 2) did not change the gross characteristics of their firing patterns after application of apamin. Most important, type II neurons could not be converted to bursting cells by this treatment, which clearly separates them from the “regular firing cells” described by Aizenman and Linden (1999) (see DISCUSSION).

**Action potential parameters and afterpolarizations**

In addition to the ability to generate bursts, prominent differences in the form of the action potential and its afterpolarizations were observed between type I and type II neurons (Fig. 7). As measured from spontaneous action potentials (no bias 5C), the acceleration–deceleration pattern appeared in a graded fashion. In type II neurons, firing frequency increased with higher current amplitudes but an acceleration–deceleration pattern could never be evoked (not even with the highest current amplitudes applied) (Fig. 5D). Similar results to those shown in Fig. 5 were found for all type I neurons (fast adaptation, $n = 32$; acceleration–deceleration, $n = 10$) and all type II neurons ($n = 7$).

Type I and II neurons also differed with respect to a slow afterhyperpolarization that was present in type I neurons after long spike trains (e.g., bursts) but that was absent in type II neurons. We use the term burst-AHP to distinguish this slow afterhyperpolarization from the faster AHPs seen after each action potential. Figure 6 shows examples of long depolarizing pulses applied to cells of both types and their effects on membrane potential after pulse offset. Both types of neurons showed a burst-AHP if the prestimulus potential was more negative than −60 mV. However, elevating the potential above that level abolished burst-AHPs in type II neurons whereas in type I neurons they were still visible (Fig. 6, $A_1$ and $B_1$). Consistent with the idea that burst-AHPs in type II neurons can be fully explained by the activation of $I_{HT}$, the burst-AHP was blocked by cesium ($n = 4$, Fig. 6B2). Therefore, the burst-AHP most probably reflects the inactivation of the delayed rectifier during the long current pulse and its subsequent activation by hyperpolarization of the prestimulus potentials after pulse offset, as demonstrated by Bal and McCormick (1997) in inferior olive neurons. In type I neurons, in contrast, cesium extended
current injected), action potential width and amplitude were significantly larger in type II neurons than they were in type I neurons (type I width, 1.52 ± 0.59 ms; type II width, 2.11 ± 0.45 ms; type I amplitude, 66.7 ± 10.4 mV; type II amplitude, 78.0 ± 8.6 mV; see Table 1). Moreover, the form of the afterpolarizations exhibited by both types of neurons was more complex in type I neurons than it was in type II neurons. Type I neurons showed a sequence of afterpolarizations consisting of an initial fast afterhyperpolarization (smaller than 1 ms) (Fig. 7A, a) followed by an afterdepolarization (ADP; peak 4.8 ± 1.8 ms after the peak of the action potential, n = 55) (Fig. 7A, b) and a shallow AHP that led to the next action potential (in Fig. 7A, c). The expression of this sequence was dependent on the circumstances of the generation of the action potential. It was best seen during spontaneous activity (without bias current injection) (Fig. 7A) and at the end of a burst (Fig. 7B, ○). In the beginning (+) or middle (+) of a burst, when instantaneous firing rate and/or action potential width was highest, this sequence typically became invisible. Only 8 of 63 type I neurons did not show this sequence of afterpolarizations and instead exhibited an isolated AHP. The AHP in these cases was shallow and showed a kink in the waveform instead of a clear ADP (not shown). Type II neurons showed a single deep AHP characterized by a lower minimum potential (Fig. 7A) that was easily distinguished from the AHP seen in all type I neurons. To quantify this difference, we measured the potential at the maximum ADP for type I neurons. Because DC offsets might be critical when small differences in potential are compared, we expressed the ADP amplitude relative to the firing threshold. The firing threshold was chosen as a reference because it did not differ significantly between the two types of neurons (Table 1). Afterpolarizations of type I and type II neurons were analyzed with an algorithm that detected a local maximum 8–15 ms after the action potential. The measurement taken from type II neurons was in all cases equal to the potential reached 8 ms after the action potential because the potentials of all type II neurons decayed monotonically within the analyzed interval. The resulting parameters showed a clear and significant difference between the two types of neurons (type I, 7.46 ± 6 mV; type II, 16.98 ± 4.1 mV) (see Table 1). Treatment of both types of neurons with apamin, a blocker of the SK-type of calcium-dependent potassium channels (Blatz and Magleby 1986), resulted in a clear change in the sequence of afterpolarizations of type I neurons whereas the AHP of type II neurons was virtually unchanged (Fig. 8). In type I neurons, the AHP–ADP–AHP sequence was entirely blocked and the firing frequency was substantially increased (Fig. 8, A1 and A3, n = 3). Extending an interspike interval recorded under apamin to the same length of one observed under control conditions shows the detail of change from the complex sequence to a smooth transition to the next spike (Fig. 8A2). In type II neurons that were depolarized with pulses of comparable strength and prestimulus potential, only a small increase in firing frequency was detectable (Fig. 8, B1 and B3; n = 2). However, the waveform of the interspike interval stayed essentially the same (Fig. 8B2).

We next investigated if burst firing of type I neurons was caused by specific factors of the patch clamp method, such as recording at room temperature. Raising the temperature in the bath to 33°C did not reveal principal differences in burst firing as compared with room temperature. The instantaneous firing rate during the burst was found to be increased from 13.9 ± 0.1 to 22.8 ± 1.7 spikes/s (n = 2). To address remaining doubts that bursting might be an artifact of the patch clamp method, we resorted to standard tungsten electrodes to record extracellularly from CN neurons in the slice. We expected that extracellular recordings would be able to reveal examples of burst firing because some intracellularly recorded neurons showed burst firing even without intracellular current injection. Indeed, the general firing patterns, as defined intracellularly, also were found in the extracellular recordings (Fig. 9). Most of the cells recorded extracellularly were continuously active, with frequencies comparable to those assessed with intracellular recordings (extracellular, 10.9 ± 3.1 spikes/s, n = 6; intracellular, 12.8 ± 6.1, n = 54). Regular spike trains assessed using intracellular and extracellular recording configurations showed a high degree of rhythmicity. Autocorrelograms showed 6.9 ± 7.9 significant side peaks (n = 54) in intracellularly recorded spike trains and 8.2 ± 4.6 significant side peaks (n = 6) in extracellularly recorded spike trains (Fig. 9, A1 and A2). In 4 of 10 cases, burst firing was present in extracellular recordings (Fig. 9B2). Although the frequency of occurrence and the length of these extracellularly recorded bursts were well within the range found with intracellular recordings, burst firing as observed with the whole cell patch method was, on average, slower and more regular. Figure 9, B1 and B2, depicts two typical examples of burst firing taken from an intracellular recording (without bias current) and an extracellular recording. The patch clamp method revealed a mean frequency of 0.04–
0.64 bursts/s \((n = 63)\) whereas the burst frequency in extra-
cellular recordings ranged between 0.4 and 1.04 bursts/s \((n = 4)\). Within the extracellular recordings, only two of four cells showed significant side peaks (one showed 3 and the other showed 4 significant side peaks). Traces of intracellular recordings, which were long enough so that at least five bursts could be assessed, showed 1.2 \pm 0.9 \((n = 41)\) significant side peaks. In summary, these findings indicate that burst firing per se was not an artifact caused by the use of whole-cell patch recordings. Using the whole-cell patch method, however, made burst firing more rhythmic and decreased the frequency of bursts.

**Morphology**

Thirty-nine of sixty-three type I neurons and two of seven type II neurons could be filled with neurobiotin sufficiently well to reveal their dendritic morphology. Almost all of the stained neurons were located within the lateral nucleus. In a few cases, however, it remains uncertain whether cells were located at the border of or within the interposed nucleus because nuclear borders could not be determined with sufficient precision in the depth of the parasagittal slices. As shown in Fig. 10, type I neurons showed a large variation in their dendritic features. The area of their somata and the maximal diameter of their dendritic trees ranged from 133 to 672 μm² and 240 to 561 μm, respectively (Fig. 10B), which covered the whole spectrum of soma sizes demonstrated by Golgi stain techniques (Chan-Palay 1977). Dendritic trees were of variable forms and showed 2–8 basal dendrites. The proximal dendrites branched into thin distal dendrites covered with a few filiform dendritic appendages of different length, some of which resembled spines. Similar to the Golgi study of Chan-Palay (1977), our results showed that the appendages were unevenly distributed and sometimes appeared to be clustered. Axons were less reliably stained in the slice. In four type I neurons, an axon could be demonstrated that left the confines of the CN, which indicates that these cells were projection neurons (Fig. 10A). In two of these cases, the main axon extended into the white matter of the cerebellar cortex but the synaptic ending could not be traced. In the other two cases, the axon ran in the direction of the fourth ventricle. Of 33 axons that could be traced within the CN, 27 showed intrinsic collaterals (Fig. 10A, arrow). Only one of the six axons that did not show collaterals could be traced beyond the borders of the CN. Most of the collaterals left the main axon close to the soma but some were...
observed to branch at points outside the immediate vicinity of the cell. Interestingly, in many cases, one of the collaterals could be traced back to the region where the cell’s own dendritic tree was localized (Fig. 10A, arrow). Infrared video microscopy allowed us to conclude that type II neurons were, without exception, small. However, because of the small size of type II neurons, it was difficult to stain them intracellularly, as discussed in Methods. Therefore, we proceeded in demonstrating the dendritic morphology in only two cases. Even in these two cells, some of the dendrites had to be classified as being cut by the slice procedure (Fig. 10A, arrowheads). Both stained type II neurons displayed a soma that was among the smallest within the sample (140 and 130 μm², Fig. 10B). Furthermore, the dendritic tree was in the smallest range of CN neurons and, when compared with type I neurons, was clearly rarified. In both cells, three basal dendrites emerged from the soma at opposing sides. The basal dendrites branched into a few thin, beaded, small-diameter dendrites that did not bear any dendritic appendages.

Statistics

A major difference between the types of neurons is the existence of long depolarizing potentials. These potentials are confined to type I neurons and are based on low- and high-threshold calcium action potentials, which are not present in type II neurons. Correlated with the ability to generate bursts, several action potential parameters, such as firing frequency, action potential amplitude, action potential width, and the form of afterpolarizations, were found to be clearly distinct between the two groups. We performed a multivariate statistical analysis to further consolidate the notion that there are two types of neurons. We chose 10 variables that capture the essential features of the CN neurons studied. The age of the animal was included to control for the possibility that burst firing is an artifact of development. We then included some parameters that are dependent on the characteristics of the membrane, the size of the neuron, and the recording configuration (e.g., the attachment of the pipette to the membrane). Such variables were the input resistance and the time constant as measured at −60 to −65 mV with test pulses of 5 pA. We next included several parameters that describe the action potential and its afterpolarizations: the action potential amplitude (measured as the difference between the action potential peak and the minimum of the AHP following it), the action potential width (measured at half action potential amplitude), the maximal slope during the upstroke of the action potentials, the firing threshold, and the ADP amplitude. Finally, we included variables that describe the pattern of firing: the spontaneous firing rate (using no bias current) and the number of action potentials generated after the offset of a 300-ms depolarizing current pulse (“plateau discharge”; see Fig. 2). We performed a MANOVA to test the hypothesis that the two types of neurons show significant differences in these parameters. This approach seemed feasible because the distribution of 8 of 10 parameters did not significantly deviate from normal (Kolmogorov-Smirnov test, \(P > 0.05\)). The two remaining variables, the input resistance and the plateau discharge of type I neurons, showed a slight deviation from a normal distribution (input resistance, \(P = 0.02\); plateau discharge, \(P = 0.02\)). The MANOVA (Table 1) indicated a highly significant difference \((P < 0.000031)\) within the means of the multivariate data. Post-hoc Scheffé tests revealed significant differences in action potential width \((P < 0.01)\), action potential amplitude \((P < 0.02)\), relative ADP amplitude \((P < 0.001)\), and instantaneous firing rate \((P < 0.02)\). No significant differences were found for age \((P > 0.55)\), time constant \((P > 0.51)\), and maximal slope of action potential \((P > 0.72)\). For the non-normal distributed variables, we applied the non-parametric Mann-Whitney test. Both of these variables (the input resistance \((P < 0.007)\) and the number of action potentials during plateau discharge \((P < 0.01)\)) showed highly significant differences.

Discussion

The present study provides the first evidence for the existence of two groups of cells in the CN based on their intrinsic membrane properties and morphological characteristics. The hallmark of the first type (type I), which comprises neurons with a large variety of morphological parameters, is the presence of plateau potentials along with a strong tendency to fire cyclic bursts of action potentials on constant hyperpolarization. The other group of CN neurons (type II) lacks both cyclic bursts and plateau potentials and displayed the smallest size of somas and dendritic fields.

Role of the patch clamp technique for the detection of two neuronal types in the CN: methodological considerations

With respect to the population of type I cells, our whole-cell patch recordings revealed a larger variety of membrane properties than was previously known from recordings with high-resistance electrodes. In addition to the demonstration of all potentials described so far (Aizenman and Linden 1999a; Jahnsen 1986a; Llinás and Mühlethaler 1988b), we found new ones that were not described before: sequences of afterpolarizations following each spike as well as slow burst-AHPs. Moreover, low-threshold calcium action potentials (Llinás and Mühlethaler 1988a,b) and the delayed rectifier (Jahnsen 1986a), which were described in one study but not in another, were observed with higher consistency in the present study [compare Fig. 3 in Jahnsen (1986a), Figs. 5 and 6 in Llinás and Mühlethaler (1988a), and Fig. 2 in Llinás and Mühlethaler (1988b)]. Aizenman and Linden (1999) detected both a low-threshold spike and a delayed rectifier but, compared with the present results, to a lesser extent. An important factor contributing to the higher consistency of patch clamp recordings in revealing these conductances may be the higher input resistances yielded by patch pipettes as compared with those obtained with high-resistance microelectrodes [608 ± 508 MΩ in the present study, 51 ± 3 MΩ in Aizenman and Linden (1999), 44 ± 10 MΩ in Jahnsen (1986a), and 24 ± 5 MΩ in Llinás and Mühlethaler (1988a)]. High-input resistance may allow small membrane currents (or those with a small influence on the somatic membrane potential) to reach a sufficient effect and therefore to generate cyclic burst firing. On the other hand, the possibility of visualizing neurons and specifically aiming for selected ones has been decisive for the detection and assessment of membrane characteristics of the small type II neurons.

A concern with the whole-cell patch method is the rapid exchange of intracellular fluid by the pipette solution. Therefore, the intracellular composition of intracellular fluid and,
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most importantly, its content of second messenger substances presumably differs substantially from what is present in recordings using high-resistance microelectrodes in which the wash-in of pipette solution is much slower. In the present experiments, a standard concentration of the calcium chelator EGTA (5 mM) was used to buffer calcium. The fact that at least two calcium-dependent AHPs (burst-AHP and fast apamin-sensitive AHP) (Blatz and Magleby 1986; Sah 1996) could be demonstrated in the presence of 5 mM EGTA indicates that the effects of intracellular calcium were not blocked by this measure. Parallel with the exchange of intracellular fluid, a possible rundown of conductances has been described (Sakmann 1995). Although rundown of conductances cannot be generally excluded for our recordings, it is not likely to be at the base of our present finding of type II characteristics. First, rundown effects as the basis for type II properties is inconsistent with the finding of an additional membrane property in type II neurons. The apamin-insensitive AHP found in type II neurons is presumably based on ionic currents unique to these cells and thus is a supplementary feature that cannot easily be explained by a lack of conductances. Second, the good correlation of burst firing to the morphological features found for type I and II cells renders an exclusive role of rundown effects for type II characteristics unlikely. Third, features typical for type II neurons were in all cases displayed immediately after the whole-cell patch configuration was accomplished; we never observed a switch from initial bursting to non-bursting behavior (or vice versa) as would be expected if these characteristics were generated by washout effects. Furthermore, the opposite view—that the burst firing seen in type I neurons, and not its absence, represents an artifact of the intracellular fluid exchange—is refuted by the fact that we were able to use extracellular recordings to find burst behavior. It has to be kept in mind, however, that the different temporal structures of burst firing found with extracellular and intracellular recordings indicates that the environment created by the whole-cell patch clamp clearly modifies the temporal parameters of burst firing. In summary, we suggest that the whole-cell patch method reveals and temporally modifies a firing pattern that exists in vitro and therefore is not per se an artificial pattern generated by the method.

Two types of neurons rather than a continuum?

One major argument for two classes as opposed to a continuum of membrane properties is the fact that several distinct potentials carried by specific membrane currents occurred exclusively in each group. Plateau potentials, low-threshold calcium spikes, burst-AHPs, and an afterpolarization sequence following each action potential were correlated with the cyclic bursts whereas a lack of these potentials and a deep AHP following each spike characterized non-bursting cells. Some of the membrane potentials characteristic of type I neurons can easily be interpreted as contributing to the ability of these neurons to fire bursts. In this category are plateau potentials, low-threshold action potentials, the delayed rectification, and burst-AHPs. However, the clear differences in action potential parameters and afterpolarizations, which were found to be significantly correlated to the occurrence of spontaneous burst firing (Table 1) without showing a direct causal relationship to the generation of bursts, strongly support two classes of neurons instead of a continuum. The morphology of the two types of neurons, as revealed by intracellular filling, does not in itself prove the existence of two separate classes but is consistent with it because the sizes of the somata and dendritic fields of type II neurons were limited to the smallest range found in CN cells.

A recent study that used high-resistance microelectrodes to record CN cells (Aizenman and Linden 1999) reported that “regular firing” neurons could be converted to “bursting” neurons by blockade of apamin-sensitive, calcium-dependent potassium currents. Because these authors recorded from neurons displaying the largest somata within the population of CN neurons (Aizenman et al. 1999), we suggest that their “regular firing” neurons are not equivalent to the type II neurons reported here because the latter were found exclusively by aiming at the smallest neurons under visual control. Furthermore, we could not demonstrate that type II neurons are converted to bursting neurons by apamin. On the other hand, the cyclic bursts of type I neurons became stronger under apamin, which points to the possibility that the “regular firing” neurons recorded in earlier studies that used conventional recording methods (Aizenman and Linden 1999; Gardette et al. 1985; Jahnson 1986a,b; Llinás and Mülfahler 1988b) were all equivalent to the type I neurons described here. As discussed in the previous section, we suggest that the detection of cyclic burst firing in CN neurons is at least partly dependent on the intracellular recording method used.

Relationship of electrophysiologically defined neuronal types with classical ones

Recently, the neurons in the CN were grouped into three classes based on their projection target and the transmitter used. The first class comprises excitatory neurons projecting to motor centers in the brain stem, mesencephalon, and thalamus as well as feedback projection to precerebellar nuclei in the mossy fiber pathway. This class of neurons has been reported to display varying soma sizes. Cells with the largest somata, as well as those with medium to small somata, are within this group (Batini et al. 1992; Chan-Palay 1977) and have been reported to use glutamate as a transmitter (Schwarz and Schmitz 1997; Verveer et al. 1997). The second class of neurons consists of inhibitory projection neurons that target the inferior olive. These neurons have been reported to display medium to small somata and to use GABA as a transmitter (De Zeeuw et al. 1989; Teune et al. 1998). The third class of neurons has been described as the smallest of the populations of cells in the CN. These neurons are believed to distribute their axonal terminals exclusively within the CN (Chan-Palay 1977). It is important to know if the borders of these classes are congruent with one of the types defined in the present study on grounds of intrinsic properties. The sizes of the somata and dendritic fields of type I neurons that were filled and stained with neurobiotin were found to be within the total range of sizes found in the CN (Chan-Palay 1977), including very large cells. We therefore can safely conclude that at least some of the glutamatergic projection neurons are type I. Morphological information on type II neurons was scarce because only two cells could be filled. These two cells were in the smallest range of neurons found. Three additional observations from type II cells supported the notion that these neurons are among the
smallest. First, they showed significantly higher input resistances as compared with type I neurons, which points to a smaller size because input resistance is inversely related to the area of membrane reached electrotonically by intracellularly injected current (Fig. 10). Second, extraction from the slice by the forces connecting the tip of the retracting pipette to the cell’s membrane was never observed in larger neurons and speaks in favor of very small and/or delicate dendritic trees that do not anchor the cells within the tissue. Third, using interference contrast optics to visually inspect the somata during recording unequivocally showed that all type II neurons belong to the cells with the smallest somata. In fact, as mentioned in Results, most of our type II cells were recorded only after we aimed exclusively at the smallest cells found in the slice. At present, the assignment of electrophysiological types to classifications based on morphological criteria has to remain speculative although a comparison with the classification of Chan-Palay (1977) suggests that the fusiform small soma and the small dendritic field, which originates on two sides of the soma of the two type II neurons, are comparable to those that have been described for putative inhibitory interneurons. A final assignment of electrophysiological types to morphologically defined classes, however, has to await further experiments combining electrophysiological recordings with assessment of a cell’s transmitter content or projection target.

Functional role of burst firing

What might be the functional role of burst firing of type I neurons on hyperpolarizing stimuli? One possibility is that burst firing is a feature arising from a specific adaptation of CN neurons to the massive Purkinje cell inhibitory input. This inhibitory projection is unique in its numbers of fibers, the high amount of convergence, and the high-frequency firing of the presynaptic Purkinje cells and the postsynaptic CN neurons in vivo (MacKay 1988a,b; Thach 1970). The results presented here offer a possible answer to the question of how CN neurons generate high-frequency firing rates in this massively inhibitory environment: they do not cease to fire under strong tonic hyperpolarization but convert it into a distinct firing pattern. This unique feature is generated by the interaction of several membrane conductances: plateau potentials, slow calcium-dependent AHP terminating the bursts (burst-AHP), and a delayed rectifier that brings the membrane potential back to the threshold for plateaus. An additional factor is the lack of strong outwardly rectifying conductances, which were reported to impede long depolarizing potentials in other CNS neurons (Schwarz et al. 1997; Stafstrom et al. 1985; Turner et al. 1997). The crucial question is, do these membrane conductances interact in vivo in the same way? Studies on CN neurons recorded from awake behaving animals usually do not show conspicuous rebound plateaus or cyclic bursts as salient features in the spike trains of CN cells [see, for example, MacKay (1988a,b) and Thach (1970)]. However, extracellular recordings from the CN of decerebrate cats and intracellular recordings form the CN of anesthetized cats demonstrated that rebound plateaus could be evoked in CN neurons after the stimulation of cerebellar afferents (Eccles et al. 1974a,b; Kitai et al. 1977; McCrea et al. 1977). Moreover, computer simulation of a whole cerebellar network showed that the rebound activity in the CN could play a key role in cerebellar functions such as storing and recall of spatiotemporal patterns of neuronal activity (Kistler and van Hemmen 1999). Therefore, although the evidence is still scant, burst firing in CN neurons seems to be present in vivo and seems to be determined not only by intrinsic membrane properties but also by the action of pre- and intracerebellar networks. It will be important for future studies of the CN to elucidate how the membrane conductances described in the present study interact with synaptic inputs to generate bursts.

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