Sodium Imaging of Climbing Fiber Innervation Fields in Developing Mouse Purkinje Cells

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1Laboratory for Neuronal Circuit Dynamics, Brain Science Institute, RIKEN, 2–1 Hirosawa, Wako-Shi, Saitama 351-0198, Japan; and 2Department of Neuroscience and Rita Montalcini Centre for Brain Repair, University of Turin, I-10125 Turin, Italy

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Scello, Bibiana, Piergiorgio Strata, and Thomas Knöpfel. Sodium imaging of climbing fiber innervation fields in developing mouse Purkinje cells. J Neurophysiol 89: 2555–2563, 2003. First published January 29, 2003; 10.1152/jn.00884.2002. Maturation of specific neuronal connections in the mature nervous system includes elimination of redundant synapses formed earlier during development. In the cerebellum of adult animals, each Purkinje cell (PC) is innervated by a single climbing fiber (CF). In early postnatal development each PC is innervated by multiple CFs and elimination of synapses formed by supernumerary CFs occurs until monoinnervation is established at around postnatal day 20 (P20) in mice. It is not clear whether multiple CFs, or only a single CF, translocate from the cell body of immature PCs to the developing dendrite and, in case several CFs translocate, whether they share or segregate their innervation fields. To localize CF innervation fields, we imaged changes in postsynaptic sodium concentration resulting from CF-mediated postsynaptic currents. We found that more than one CF translocates from an innervation field on the cell body of the PC to the developing dendrite and that these CFs share rather than segregate their innervation fields. We concluded that both the soma and the proximal dendrite of the PC are territories of competition for the developing CFs and that the overlapping of their termination fields may be the prerequisite for a local process of elimination of all but one CF, as previously demonstrated in the developing neuromuscular junction.

INTRODUCTION

Establishment of specific neuronal connections involves elimination of redundant synapses formed during early development (Goodman and Shatz 1993; Katz and Shatz 1996; Nguyen and Lichtman 1996). The contacts between climbing fibers (CFs) and Purkinje cells (PCs) are a powerful model to study principles underlying such developmental synapse elimination. In adult animals, each PC is innervated by a single CF. However, in early postnatal development, each PC is innervated by multiple CFs and elimination of synapses formed by supernumerary CFs occurs until monoinnervation is established. Morphological and electrophysiological data suggest that, in rats, the first contacts between CF terminals and PCs are already established during embryonic life (Morara et al. 2001) and functional synapses appear at postnatal day 2 (P2) (Crepel et al. 1981). At P5, virtually all PCs are multiply innervated with, on the average, 3.5 CFs impinging on each PC (Crepel et al. 1981). At this developmental stage, when PCs are morphologically immature with only rudimentary dendrites, the initial contacts between CFs and PCs are made at the perisomatic level (Altman and Bayer 1997). Regression of multiple innervation starts at P5 (Crepel et al. 1981) and it could be argued that confining inputs to the limited space of the cell soma enhances a competitive process, resulting in elimination of supernumerary CFs (Hume and Purves 1981; Purves and Hume 1981). By P9 the alignment of PCs into a monolayer is completed. At this stage the dendritic tree of the PC starts showing its typical appearance: a single-stem dendrite extends into the molecular layer and divides into many branches from which tertiary and distal branches emerge. By P13 PC morphology is reminiscent of that of adult animals (Mason et al. 1990). During this time window CF branches gradually move to the growing dendrite and complete their translocation to attain their final innervation at the proximal portion of the dendrite.

Parallel fibers (PFs), the second excitatory input to Purkinje cells, are required for normal growth of the dendrite and they make contact with distal dendrites after P7 (Altman 1972; Sotelo 1978). In addition, PFs play a role in the regression of multiple innervation by CFs. In fact, when granule cells are deleted, have degenerated (Bravin et al. 1995; Crepel et al. 1981; Mariani et al. 1990), are impaired in their function (Rabacchi et al. 1992), or their transduction pathways are deficient (Conquet et al. 1994; Hashimoto et al. 2001a,b; Kano et al. 1995, 1997, 1998), the regression of the CF multiple innervation is hampered. It is known that, in the cerebellum made hypogranular during postnatal development, different CFs can translocate and occupy separate dendritic territories of adult PCs (Bravin et al. 1995; Zagrebelsky and Rossi 1999). However, in mice with normal development, it is not known whether the presence of normal PFs prevents the translocation of more than one CF from the soma to the dendrites or whether competition between CFs occurs, at least in part, at the dendritic level after more than one CF has translocated. In the case of multiple translocation, a second question is whether the innervation territory is separated or overlapping.

We addressed this question using sodium imaging techniques in slices prepared from mice at developmental stages ranging from P6 to P20. In contrast to calcium imaging techniques that rely on activation of voltage-gated Ca2+ channels
and hence reflect the spread of voltage from the site of the synaptic contacts throughout the dendritic arborization, changes in intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) represent, under the conditions employed, the flux of Na\(^+\) through synaptic glutamate receptors and are confined to the innervation fields.

**METHODS**

*Slice preparation and patch-clamp recordings*

Cerebellar slices were prepared from ICR mice aged between P6 and P14 according to previously established techniques (Edwards et al. 1989). Briefly, animals were anesthetized (in ice when aged between P6 and P7, with ether from P8 on) and decapitated. The cerebellar vermis was removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 118 NaCl, 3 KCl, 1 MgCl\(_2\), 1.5 CaCl\(_2\), 1 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 10 d-glucose. The solution was continuously gassed with 95% O\(_2\) and 5% CO\(_2\), resulting in a pH of 7.4. Parasagittal cerebellar slices (300 \(\mu\)m thick) were cut using a vibratome (Leica VT1000S) and incubated in ACSF at 35°C for the first hour and then at 25°C for \(\leq 6\) h. After at least 1 h of incubation, one slice was transferred into a recording chamber and continuously superfused with ACSF (24–26°C, 2 ml/min). Whole-cell patch-clamp recordings were obtained from PC somata with pipettes prepared from borosilicate glass and having resistances of 2.5–3 MΩ when filled with intracellular solutions consisting of (in mM) 131 CsCl, 20 TEA, 10 HEPES, 0.5 EGTA, 0.1 CaCl\(_2\), 0.4 Na-GTP, 4 Na-ATP, 5 QX314, and 2 SBFI (tetraammonium salt, Molecular Probes, Eugene, OR), pH 7.3. Glass pipettes pulled from sodalime glass (tip diameter 3–10 \(\mu\)m) and filled with extracellular solution were used for electrical stimulation (negative current pulses, 5 to 100 \(\mu\)A, 300 ms). Synaptic responses were recorded in ACSF containing 20 \(\mu\)M bicuculline methiodide and 50 \(\mu\)M d-2-amino-5-phosphono-pentanoic acid (d-APV).

**Imaging**

Fluorescence of SBFI was excited by epillumination with light provided by a monochromator (Polychrome II, Till Photonics, Germany) and detected by a cooled 12-bit charge coupled device (CCD) at 4–5 Hz under control of Axon Imaging software (Axon Instruments, Forster City, CA) as previously described (Knöpfel et al. 2000). Fluorescence images were corrected for background fluorescence (measured from image regions free of dye). At the excitation intensities employed, photobleaching of SBFI was small (<0.05%/s) and was corrected for by using control recordings without stimulation. Changes of [Na\(^+\)]\(_i\), were expressed as relative fluorescence changes (\(\Delta F/F\) values) as described previously (Muri and Knöpfel 1994).
Color-coded maps of $\Delta F/\Delta F$ were obtained from the change in fluorescence measured during the first second following onset of the CF stimulation using custom-made macros under IDL 5.2 (Research Systems) and Image-Pro Plus (Media Cybernetics). $\Delta F/\Delta F$ values are unreliable in regions where the absolute baseline fluorescence level ($F$) approaches zero (i.e., at the border of the cells) and $\Delta F/\Delta F$ values are undefined when $F$ reaches zero. Therefore a masking technique was employed in which the brightness of each pixel of the $\Delta F/\Delta F$ maps was derived from the corresponding $F$ value. Therefore regions exhibiting no dye fluorescence ($F = 0$) are black, and dim structures, such as very fine processes, are in darkened colors. In images in which the color scale ranges from 0% to a maximal $\Delta F/\Delta F$ level (see Figs. 3, B, C, and E; Fig. 5, B–D, G, and H; Fig. 6, B–D; and Fig. 7, B and C), nonresponsive areas of the cells are represented in the color corresponding to zero $\Delta F/\Delta F$ values (i.e., bluish colors). In some images (Figs. 3, G–I and 4A) the color scale was limited to a range starting from a threshold value above zero. Pixels with $\Delta F/\Delta F$ values below this threshold represent the corresponding $F$ value in gray scale to indicate the cell’s morphology.

RESULTS

The morphology of postnatal development of rat and mouse PCs has been well described (Altman 1972; Eccles 1970). SBFI-filled mouse PCs between P6 and P14 exhibit a remarkable degree of variability within the same age, and the development of the dendritic tree displays rapid maturation between P6 and P9 (Fig. 1).

To study responses mediated by individual CFs, we first established a rigorous scheme for their identification. CF-mediated excitatory postsynaptic currents (EPSCs) were first identified by their all–or–none nature in responses to a stimulus of graded intensity and by their feature of paired-pulse depression (Eccles et al. 1966). Multiple CFs were recruited in some initial experiments with one stimulation electrode, after establishing multiple all–or–none thresholds of stimulation intensity (Crepel et al. 1976), but in the majority of experiments two stimulating electrodes were used. When using two stimulation electrodes, each stimulation pipette was moved systematically in the granule cell layer until an isolated all–or–none CF response was recruited at minimal stimulation intensity. Higher stimulation intensities often recruited additional CF responses but stimulation at these intensities was not used. The use of two stimulating electrodes allowed us to confirm that isolated individual CF responses, when activated together, sum up and do not depress each other when stimulated sequentially within a short time window, as expected if the same CF was stimulated by the two stimulation electrodes (see Fig. 2, C and D).

Previous experiments illustrated that CF stimulation induces relatively small changes in $[\text{Na}^+]_i$ (Callaway and Ross 1997; Lasser-Ross and Ross 1992) and, consequently, only a small change in fluorescence of the sodium-sensitive dye SBFI. To achieve clearly detectable fluorescence signals, CFs were stimulated with a train of five stimuli at the frequency of 10 Hz. Figure 3 illustrates maps of increases in $[\text{Na}^+]_i$, obtained from a P10 PC under these conditions. Suprathreshold, but not subthreshold CF stimulation induced an elevation of $[\text{Na}^+]_i$ that was confined to the proximal dendrites (Fig. 3, A–C). The time course of changes in $[\text{Na}^+]_i$, obtained from the responsive area showed that elevated $[\text{Na}^+]_i$ decays to resting levels with a time constant of about 1 s as described previously (Fig. 3D).

FIG. 2. A: experimental design. Two stimulating electrodes (S1 and S2) were placed in the granule cell layer (GL) below the recorded Purkinje cell. Responses to climbing fiber (CF) stimulation were recorded from the soma of the Purkinje cell with a patch pipette (R). B: identification of CF responses by their all–or–nothing nature at threshold stimulation intensity and by paired pulse depression. C and D: identification of independent CF responses evoked by first (S1) and second (S2) stimulation electrode. C: simultaneous stimulation with both electrodes leads to summation of individual CF responses. D: response induced by one electrode does not induce paired pulse depression of the response induced by the other stimulation electrode.

(Knöpfel et al. 2000). The CF-mediated change in $[\text{Na}^+]_i$, was abolished by the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (Fig. 3, E and F; $n = 29$). In a set of experiments we also performed sodium imaging with PF activation (5 stimuli at 10 Hz) to compare the spatial location of the PF responses relative to that of CFs (Fig. 3, G–I; $n = 3$). PF-induced sodium changes were confined to a small portion of the distal dendrite of the PC in close vicinity of the location of the stimulation electrode (Fig. 3H). As with the CF-mediated signals, PF-mediated $[\text{Na}^+]_i$ signals were completely abolished by NBQX (20 μM) (Fig. 3I). In the majority of the experiments Purkinje cells were loaded with QX314 (5 mM), a blocker of voltage-gated sodium channels that was added to the pipette solution. QX314 abolished induced activation fast action potentials by antidromic stimulation or direct depolarization of the PC (not shown). The use of QX314 is indicated for each illustrated cell in the corresponding figure legend and in the summarizing Table 1. However, no difference was seen in CF-induced $[\text{Na}^+]_i$ responses between cells with and without QX314, indicating that the present $[\text{Na}^+]_i$ signals do not contain a component mediated by QX314-sensitive voltage-gated ion channels.

It has been shown that $\text{Na}^+$ diffuses essentially freely in the cytoplasm of Purkinje cells (Callaway and Ross 1997; Knöpfel et al. 2000). Consistent with diffusion of $\text{Na}^+$ entering the cell via AMPA receptors, the monitored rise of the $[\text{Na}^+]_i$ responses outlasted the synaptic currents for $\approx 500$ ms. Therefore
our maps of \( \Delta[Na^+] \), overestimate the actual innervation field. However, the size of \([Na^+]\), signals resulting from diffusion rapidly decline as a function of distance from the source, such that they turn out to be clearly subthreshold under the present conditions of generating the maps of \( \Delta[Na^+] \), at distances > 30 \( \mu \text{m} \) (Knöpfel et al. 2000). This upper limit of overestimation of innervation field size is also consistent with the restricted PF-mediated \([Na^+]\) signals (Fig. 3H).

All the above control experiments indicate that the maps of \( \Delta[Na^+] \) represent sodium flux through postsynaptic AMPA receptors and represent the innervation field of the activated presynaptic elements at a spatial resolution sufficient to detect segregated innervation fields as described in hypogranular or

### TABLE 1. CF innervation territories in polyinnervated PCs

<table>
<thead>
<tr>
<th>Age (postnatal days)</th>
<th>Partially Intermingled Innervation Fields</th>
<th>Fully Intermingled Innervation Fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>6–8</td>
<td>3 (2)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>9–11</td>
<td>5 (1)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>12–14</td>
<td>1 (1)</td>
<td>2 (1)</td>
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A total of 17 cells in which two CF responses could be independently evoked were analyzed. For 3 age intervals cells were classified with respect to the overlap of their innervation fields. Values are numbers of cells with number of cells that have been recorded with QX314-containing patch pipettes in parentheses. CF, climbing fibers; PCs, Purkinje cells.
some mutant mice (Bravin et al. 1995; Hashimoto et al. 2001a,b).

Translocation of climbing fibers

Using the above approach we characterized the developmental transition of CF innervation fields. Maps of CF-induced increases in \([\text{Na}^+]/\text{H}^+\) from 43 PCs at postnatal ages between P6 and P20 were divided into four age groups and classified depending on the localization of the \([\text{Na}^+]/\text{H}^+\) signals at the cell body (soma), soma and proximal dendrite, and proximal dendrite (Fig. 4). We never detected CF-induced elevations of \([\text{Na}^+]/\text{H}^+\) at the distal part of the PC dendrite, in agreement with anatomic investigations reporting that CF contacts are confined to the large dendritic branches (Mason et al. 1990; Palay and Chan-Palay 1974). The developmental profile of the CF termination field shows that CF translocation starts at P6 and is essentially completed by P11 in mice (Fig. 4B).

More than one CF translocates from the soma to the proximal dendrite

To investigate whether one or multiple CFs translocate to the dendritic target field we performed imaging experiments in PCs in which we could isolate two independent CFs. Figure 5 illustrates data obtained from P9 and P11 PCs. At this developmental stage the bulk of translocation takes place (Fig. 4). The P9 cell (Fig. 5, A–E) exhibits two CF termination fields that are most prominent at the level of the soma but both extend to the proximal dendrite. The two CFs of the P11 cell (Fig. 5, F–H) innervate the proximal dendrite while only one of them appears to have a remaining weak contact on the cell body (Fig. 5G). At P14, when translocation is completed in the majority of cells (Fig. 4B) while innervation by more than one CF is still observed (Kano et al. 1995), two CFs are fully translocated (Fig. 6).

Multiple translocated CFs share their innervation fields

The examples in Figs. 5 and 6 not only show that more than one CF can translocate onto the dendrite but also that their innervation fields are overlapping or at least are intermingled. Figure 7 shows a particular illustrative example of a P11 PC with two primary dendrites and dendritic innervation by two fully translocated CFs. One of the two primary dendrites (Fig. 7, upper dendrite) is innervated by the first CF while the second primary dendrite is innervated by both CFs. Thus the first primary dendrite represents the mature situation of a monoinnervated proximal dendrite while the second primary dendrite

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**FIG. 4.** Translocation from somatic to dendritic innervation fields of CFs during development. A: maps of CF-induced \(\Delta[\text{Na}^+]/\text{H}^+\) in P7, P9, and P12 PCs. Responsive areas (defined as \(\Delta[\text{Na}^+]/\text{H}^+\), greater than the minimal value indicated at the color bars) are superimposed on the fluorescence image of the cells. Scale bars = 20 \(\mu\)m. Recordings from the P9 and the P12 PCs were conducted with patch pipettes containing QX314. Regions of the image in which the change in SBFI fluorescence was smaller than the range given by the color scale show the fluorescence image of the cell in gray scale. B: CF territories classified as soma only, soma and proximal dendrite, and proximal dendrite only at different developmental stages.
We divided the data into three age groups and with regard to the relative location of different CFs innervation fields (see Table 1). Our data demonstrate that multiple CFs have intermingled or partially intermingled innervation fields either on the soma or on the proximal dendrite of the PC. We never detected multiple innervation where CFs have completely separate synaptic territories, supporting the conclusion that multiple CFs share rather than segregate their innervation fields on the PCs in the developing cerebellum.

**DISCUSSION**

We employed electrophysiological and imaging techniques to investigate the CF synaptic termination fields in cerebellar cortex of mice during the period in which developmental synapse elimination converts multiple CF innervation to monoinnervation (Kano et al. 1995). We found that more than one CF can translocate from the cell body to the proximal dendrite and that translocated dendritic CFs have intermingled terminal arbors.

Our data of the developmental profile of CF termination fields confirms that CF translocation lags behind outgrowth of the dendrite (Mason et al. 1990). In fact, already at P8 the PC dendrites display a significant degree of development (see Fig. 1) while at the same time period CF innervation is mainly confined to the perisomatic level (Fig. 4) (Mason et al. 1990). These findings are in agreement with the view that PC dendritic development is not strictly dependent on CFs (Mason et al. 1990) but under the influence of the PFs. In fact, in several
experimental conditions in which granule cells are deleted, PC dendrites are atrophic and disoriented in space (Altman and Anderson 1972; Berry and Bradley 1976; Bradley and Berry 1978; Bravin et al. 1995; Caviness and Rakic 1978; Crepel et al. 1980; Mariani et al. 1977; Sotelo 1978; Woodward et al. 1975). Experiments aimed to test the influence of CFs on PC dendritic development have shown that dendritic growth can occur in the absence of CF input (Calvet et al. 1976; Sotelo and Arsenio-Nunes 1976).

Although it is clearly established that regression from polyto monoinnervation is hampered when granule cell function is abnormal, this information does not answer the question of whether, in normal conditions, the presence of functionally active PFS conveys to the dendrites signals to prevent the invasion of more than one CF. Indeed, we showed that more than one CF invades the dendritic tree, indicating that normal PFS do not prevent a transient innervation of the PC dendrites by multiple CFs and that at least part of the competition occurs on the dendritic territory.

Imaging experiments performed on polyinnervated PCs allowed us not only to follow the process of translocation but also to study the relative allocation of CF innervation fields. In all cases of multiple dendritic innervation we found that CF termination territories are overlapping. The situation is similar to that described at the neuromuscular junction, which exhibits a variety of similar features. Also, in the latter system, a transient phase of polyinnervation is followed by a permanent state of monoinnervation, and perturbation of this process is associated with functional deficits. Many studies, in vivo and in vitro, have shown that regression of redundant connections is an activity-dependent process involving pre- and postsynaptic mechanisms (Dan and Poo 1992; Lo and Poo 1991). Morphological studies involving in situ imaging of polyinnervated muscle fiber revealed that, at birth, terminal branches of different axons are completely intermingled. However, during several weeks after birth, the termination fields progressively segregate before the complete withdrawal of all but one motor axon. The axon branches that innervate overlapping postsynaptic muscle cells retract first (Gan and Lichtman 1998; Keller-Peck et al. 2001). In a cell culture system containing motoneurons and myocytes, it was shown that brief tetanic stimulation of one neuron resulted in immediate functional suppression of unstimulated axons innervating the same muscle cell only and only if innervation fields of the interacting axons were spatially separated by <50 μm (Lo and Poo 1991). Thus the degree of separation of different innervation sites can be a determining factor in the process of synapse elimination. Such a scheme would explain the finding that in hypogranular cerebellum and mice lacking glutamate receptor δ2 subunit or metabotropic glutamate receptor subtype 1, where PC remain polyinnervated in adults, CF innervation territories are segregated (Bravin et al. 1995; Hashimoto et al. 2001a) while, as shown in

**FIG. 6.** Multiple dendritic CF innervation in a P14 PC. Two individual climbing fibers were stimulated with two separated electrodes. A: fluorescence image of the cell. Scale bar = 30 μm. B–D: color-coded maps of Δ[Na⁺], and time courses of membrane currents and Δ[Na⁺], obtained with stimulation of the first CF (B), the second CF (C), and concurrent stimulation of both CFs (D). Δ[Na⁺] traces were obtained from the responsive area indicated in A. E: lack of mutual paired pulse depression. Note that translocation has been completed for both the CF and the territories of innervation are intermingled on the proximal dendrite but are separated on the secondary dendrite.
this work, lack of CF segregation in normal cerebellar development facilitates competition between developing CF terminal arbors and ultimately elimination of all but one CF.

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