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The Neuronal Organization of a Unique Cerebellar Specialization: The Valvula Cerebelli of a Mormyrid Fish

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Abstract

The distal valvula cerebelli is the most prominent part of the mormyrid cerebellum. It is organized in ridges of ganglionic and molecular layers, oriented perpendicular to the granular layer. We have combined intracellular recording and labelling techniques to reveal the cellular morphology of the valvula ridges in slice preparations. We have also locally ejected tracer in slices and in intact animals to examine its input fibers. The palisade dendrites and fine axon arbors of Purkinje cells are oriented in the horizontal plane of the ridge. The dendrites of basal efferent cells and large central cells are confined to the molecular layer, but are not planar. Basal efferent cell axons are thick, and join the basal bundle leaving the cerebellum. Large central cell axons are also thick, and traverse long distances in the transverse plane, with local collaterals in the ganglionic layer. Vertical cells and small central cells also have thick axons with local collaterals. The dendrites of Golgi cells are confined to the molecular layer, but their axon arbors are either confined to the granular layer or proliferate in both the granular and ganglionic layers. Dendrites of deep stellate cells are distributed in the molecular layer, with fine axon arbors in the ganglionic layer. Granule cell axons enter the molecular layer as parallel fibers without bifurcating. Climbing fibers run in the horizontal plane and terminate exclusively in the ganglionic layer. Our results confirm and extend previous studies and suggest a new concept of the circuitry of the mormyrid valvula cerebelli.

Indexing terms

Purkinje cell; efferent cell; large central cell; deep stellate cell; Golgi cell; parallel fiber; climbing fiber; valvula cerebelli; mormyrid fish

The cerebellum is an important brain structure in almost all vertebrate groups, reaching its relatively largest size and differentiation in fishes, birds and as in mammals (Nieuwenhuys et al., 1998). Throughout the diverse vertebrate radiation the cerebellum has maintained a strikingly consistent and regular structure and organization, including 1) the existence of

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three layers, i.e. a granular layer, a layer of Purkinje cells and a molecular layer; 2) divergent, excitatory mossy fiber input to glomeruli in the granular layer; 3) a transversely-oriented, excitatory parallel fiber projection from granule cells to Purkinje cell dendrites in the molecular layer; 4) sagittally-oriented spiny dendrites of Purkinje cells in the molecular layer on which parallel fibers massively synapse; 5) an inhibitory projection from Purkinje cells to cerebellar efferent cells; 6) an excitatory projection from cerebellar efferent cells to a variety of predominantly motor and premotor brain centers; 7) the presence of at least two inhibitory feedback loops in the mossy fiber-parallel fiber-Purkinje cell circuitry, established by Golgi cells in the granular layer and by stellate cells in the molecular layer; and 8) the presence of a sagittally-oriented olivocerebellar climbing fiber projection to Purkinje cells, in which each Purkinje cell receives input from only one climbing fiber (Ramon y Cajal, 1911; Ito, 1984; Nieuwenhuys et al., 1998).

In spite of a long tradition of research and a multitude of data from the mammalian cerebellum, a general concept of basic cerebellar mechanisms and function is still lacking. It is generally thought that climbing fibers, when conjunctively activated with parallel fibers, induce long-term depression (LTD) of synaptic efficacy at parallel fiber-Purkinje cell synapses, a form of plasticity considered to be a cellular mechanism for adaptive behavior and learning (Raymond et al., 1996; Kim and Thompson, 1997; Ito, 2001; Jorntell and Hansel, 2006). However, it is still unknown why the molecular layer needs an orthogonal organization for this purpose, because similar learning mechanisms occur in the mormyrid electrosensory lobe (ELL), a cerebellum-like structure in which the molecular layer lacks such organization (Bell et al., 1997; 2008; Sawtell et al., 2007). It is also unknown why it is necessary for climbing fibers to “climb” into the mammalian dendritic tree of Purkinje cells, since similar climbing fiber-parallel fiber interactions occur and lead to LTD of parallel fiber synaptic strength in the central lobes of mormyrid (Han et al., 2007), where olivocerebellar fibers terminate only on the cell bodies and proximal dendrites of Purkinje cells (Finger, 1983; Meek and Nieuwenhuys, 1991; Han et al., 2006; Meek et al., 2007). In addition, it is still unclear whether the cerebellum is predominantly involved in motor learning and control, or if it subserves sensory and cognitive brain functions as well (Paulin, 1993; Dow, 1995; Bell, 2001).

Comparative brain research has been a fruitful approach in gaining additional information and complementary views of cerebellar organization and function. An exploration of variations and specializations of cerebellar organization among vertebrates is likely to be useful in distinguishing between the basic pattern, present in all cerebellums, and the many specializations, encountered in specific animal groups and/or cerebellar subdivisions. When such variations and specializations are correlated with specific capacities, behaviours or learning mechanisms, these connections will enhance our understanding of the functional significance of specific aspects of cerebellar organization. In this respect, the valvula of mormyrids is of particular importance, since it is the most deviating cerebellar specialization encountered in vertebrates.

A valvula cerebelli occurs in the actinopterygian fishes (which include teleosts) where it is basically a rostral protrusion of the cerebellum in the midbrain ventricle (Larsell, 1967; Nieuwenhuys, 1967; Meek and Nieuwenhuys, 1997). Remarkably, the valvula is much

larger in mormyrids than in any other teleosts and covers the entire dorsal and dorso-lateral aspects of the entire brain (Nieuwenhuys and Nicholson, 1969a; Meek et al., 2007) just as the cerebral cortex covers the human brain. It also makes up as much as one percent of the total body weight (Meek, 1998), which is far more than any cerebellum or cerebellar subdivision in vertebrates. For this reason, the mormyrid cerebellum has been called a gigantocerebellum and has been attractive for neuroanatomists for over a century (Stendell, 1914; Nieuwenhuys and Nicholson, 1967; 1969a, b). It shares the general and basic organizational features outlined above with all other cerebellums and cerebellar subdivisions, but has in addition a number of striking specializations. These include: 1) general teleostean cerebellar features, such as the close location of efferent cells to Purkinje cells in the Purkinje cell (or ganglionic) layer but not to the deep cerebellar nuclei as in mammals, and the restriction of olivocerebellar “climbing” fiber terminals to the cell bodies and proximal dendrites of Purkinje cells (Meek and Nieuwenhuys, 1997; Meek et al., 2007; Han et al., 2006); 2) the mormyrid specialization of an extremely regular palisade pattern of the spiny apical dendrites of Purkinje cells in the molecular layer (Nieuwenhuys and Nicholson, 1969b; Meek and Nieuwenhuys, 1991; Han et al., 2006); 3) the presence of specialized and numerous deep stellate cells with inhibitory projections to efferent cells (Meek et al., 2007); and 4) topological transformations of the cerebellum that result in changed positions of the granular layer with respect to the molecular layer (Nieuwenhuys and Nicholson, 1969a). For example, granule cells may be located laterally to the molecular layer on both sides of the transverse plane, as in the lobus transitorius (Nieuwenhuys and Nicholson, 1969a; Meek 1992a, b), or beneath the ganglionic layer, ventral to the valvular ridges. This largest part of the valvula consists basically of a huge plate of granular layer covered with transversely-oriented ridges of molecular and ganglionic layers (Figs. 1, 2). The present study deals with the neuronal organization of this remarkable, ridged part of the mormyrid valvula.

A number of studies have been devoted to the central and caudal lobes of the mormyrid cerebellum (Nieuwenhuys and Nicholson, 1969a,b; Nieuwenhuys et al., 1974; Meek et al., 1986a,b; Meek and Nieuwenhuys, 1991; Han and Bell, 2003; Han et al., 2006; Campbell et al., 2007; Zhang and Han, 2007; Zhang et al., 2007), but detailed information on the ridged valvula is limited. As summarized in Fig. 2A, Nieuwenhuys and Nicholson (1969a,b) described the gross morphology and somatic and dendritic organization of valvular neurons using Golgi impregnation techniques. Basically, the neuronal organization of the region is similar to that of the central lobes but with a different topological arrangement. Kaiserman-Abramof and Palay (1969) studied the valvula in the electron microscope and described ultrastructural characteristics of several elements, including Purkinje cells and basal efferent cells. Another study using tract-tracing methods showed that much of the valvular ridges are directly or indirectly connected to structures related to electrosensory system (Finger et al., 1981). Physiologically, two earlier studies showed that as much as half of the valvula is associated with electrosensory processing (Bennett and Steinbach, 1969; Russell and Bell, 1978). Recently, Meek et al. (2007) studied the valvula and other subdivisions of the mormyrid cerebellum with immunohistochemical techniques. Their work revealed the abundant presence in the upper ganglionic layer of the previously unknown deep stellate cells, which have specific inhibitory projections to efferent cells. Remarkably, the latter

study also suggested that there are probably no connections between the distal part of the valvular ridges and the basally-located efferent cells.

The present study is the first step in our long term goal to understand the functional organization of the ridged part of the mormyrid valvula more completely. For this purpose we have combined intracellular recording and labelling techniques in slice preparation to characterize the synaptic and intrinsic physiological properties of valvular cells and to identify the morphology of different cell types in the valvular ridges, with special attention given to Purkinje cells, basal cells and deep stellate cells. We have also examined the morphology of parallel fibers in the valvular ridges by focal ejection of tracer in slice preparations. Finally, we have investigated the origin of the climbing fibers and their termination pattern in the valvular ridges by injection of tracer to inferior olive nuclei in *in vivo* preparations. This paper is concerned primarily with the morphology of cellular elements in the valvular ridges and with the most basic and characteristic electrophysiological features of each cell type. An immunohistochemical analysis of these cells appears in the companion paper (Meek et al., 2007). The preliminary results of this study were published in an abstract form (Shi et al., 2007).

Materials and Methods

All experiments were carried out in *in vitro* slices from the cerebellar valvula of mormyrid fish of the species *Gnathonemus petersii*. A total of 110 fish were used for these experiments, ranging in length from 6 to 15 cm. Fish were obtained from local wholesale dealers and were housed and handled according to national and institutional guidelines. All experiments were approved by the Institutional Animal Care and Use Committee of Oregon Health Science University (IACUC #0715).

Slice Preparation

The fish were deeply anesthetized with tricaine methane sulfonate (MS-222) at a concentration of 100 mg/L. The skull was opened and the brain was fully exposed and irrigated with ice-cold, artificial cerebrospinal fluid (ACSF; for composition see below). One vertical cut was done at the caudal end of the brain, and the whole brain was quickly removed and left in ice-cold ACSF for about one minute to harden the tissue. Valvular slices were then prepared in two different ways. One was to cut the whole brain transversely while it was supported by a gelatin wall; slices cut from the most rostral part of the brain are then nearly transversal through the ridges. Another way was to extract and unfold the whole valvula, which then becomes a tissue sheet of about 10×15×1 mm, depending upon the fish size. The tissue sheet can be cut into 4-6 small pieces. Individual pieces were placed on gelatin blocks and covered with liquid gelatin (12.5% gelatin is in solid form at room temperature and becomes a liquid at 30°C). The whole blocks containing the tissue were left in ice-cold ACSF for one minute to harden them. The individual blocks were then glued to a vibratome plate (Leica, TV1000, Germany) in such orientations that facilitated the cutting of transverse, sagittal or horizontal slices.

The cutting chamber was filled with ice-cold ACSF and equilibrated with 95% O₂ and 5% CO₂ during slicing. Up to this point, the ACSF contained the nonselective glutamate

receptor blocker kynurenic acid (1 mM) to reduce potential excitotoxic damage (Rossi et al., 2000). The composition of the ACSF was as follows (in mM): NaCl 124, KCl 2.0, KH_2PO_4 1.25, NaHCO_3 24, CaCl_2 2.6, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.6, glucose 20 (pH 7.3-7.5, osmolarity 295-305). After cutting, typically at 200 μm for visualized patch recordings, slices were immediately transferred into warm ACSF containing 0.5 mM kynurenic acid, and kept in a warm bath (26-28°C) for up to one hour. The slices remained in normal ACSF (without glutamate blocker) at room temperature until recording. In some cases, low-sodium ACSF was used for slicing and the NaCl was replaced by an equilibrant of sucrose (Han and Bell, 2003).

Some parts of the valvula sheet are only about 300-600 μm in thickness and can therefore be treated as a single “slice”. Cells at the top of the ridges in such slices can be readily visualized and recorded from.

Intracellular recording and labeling

In transverse slices, the laminar structure of the valvular ridges can be easily recognized under a dissecting microscope. In most cases, Purkinje cells and basal efferent cells could be visualized under infrared Nomarski optics using the 40 $\times$ water immersion objective of an upright microscope (Axoskop I, Zeiss) for whole cell patch recording (Fig. 1D). All recordings were made at room temperature (22-24°C). P-clamp 9 software (Axon Instruments, Foster City, CA) was used for data acquisition and data analysis.

The electrodes used for whole-cell patch recording had resistances of 4-7 M Ω after being filled with an internal solution that contained 0.5% biocytin or neurobiotin (Molecular Probes, Eugene, OR). The composition of the internal solution was as follows (in mM): K gluconate 130, EGTA 5, HEPES 10, KCl 3, MgCl_2 2, Na_2ATP 4, $\text{Na}_2\text{phosphocreatine}$ 5 and Na_2GTP 0.4 (pH 7.35-7.45, osmolarity 280 - 290). Cells were randomly selected from the valvular ridges under visual control. Typically, a gigaseal was formed by a gentle suction and membrane was ruptured by a small negative pressure or zap. Recordings were performed under both voltage and current clamp modes using an amplifier (Multiclamp 700B, Axon Instruments). The holding potential under voltage clamp was -70 mV unless otherwise noted. Recording was discontinued if the resting membrane potential fell below -55 mV under current clamp, or if the leak current was above 100 pA under voltage clamp.

We used a patch pipette filled with external solution for stimulation. Pulses 0.1 ms in duration and 10-100 μA in amplitude (with the electrode in the tissue negative) were delivered through a stimulus isolation unit. The ground wire was directly submerged in the recording chamber bath. The stimulating electrode was placed in the molecular layer or the granular layer to activate parallel fibers and in the ganglionic layer near the patched cells to activate climbing fibers (Fig. 2C).

After electrophysiological examination, tracer in the recording micropipettes was ejected into the recorded cells by applying tip-positive current pulses. Slices were fixed in a 4% paraformaldehyde (for fluorescent visualization) or a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde (for DAB visualization) in 0.1 M phosphate buffer (PB, pH 7.35-7.45) overnight prior to histological procedures.

Extracellular injection of biotinylated dextran amines (BDA)

Extracellular injections of BDA were made into the inferior olive nuclei in an *in vivo* preparation. The surgical preparation was performed as reported before (Bell, 1981a; Campbell et al., 2007). Briefly, fish were anaesthetized with MS-222 with the head firmly held by a rod glued to the skull. Fish were maintained in a holding tank with water passing through the gills continuously. A small hole was opened on the skull above the caudal region of the brain. The inferior olive nuclei were located by recording the field potential of the electric organ discharge (EOD) from the command nucleus, which is located in the brainstem on the midline at the anterior limit of the inferior olive nuclei and at about the same depth. The recording electrodes were filled with 10% BDA (Molecular Probes, Eugene, OR) in 0.9% NaCl. The tracer was ejected by passing DC of 2-5 μ A for ~10 min. Two to three sites were injected in each animal, and four fish were used for such injections. Animals were allowed to survive for 3 to 5 days after surgery and were then perfused through the heart with 4% paraformaldehyde in 0.1 M PB.

Extracellular injections of BDA were also made to the molecular layer and granular layer in slice preparation. Twenty-one transverse and horizontal slices of 200-300 μ m thickness were used for this purpose. Slices were prepared and handled in the same way for recording as described above. Injections were made in the same way as *in vivo* but were performed in the recording chamber under visual guide. After injection, slices were incubated in normal ACSF and kept in a warm bath for 5 to 8 hours before fixation.

Histology and cell reconstruction

Slices were routinely treated by a histology procedure. The details of the procedure are described elsewhere (Han et al., 1999; 2006). Briefly, to visualize the biocytin- or neurobiotin-labeled cells and fibers with fluorescent dyes, the slices were incubated in streptavidin-conjugated Alexa fluor 594 (1:200-500 in 0.1 M PB; Molecular Probes) for 4-6 hours, and counterstained with Neuro Tracer 500/525 (1:500 in 0.1 M PB; Molecular Probes) before being mounted in the fluorescent mounting medium Vectashield (Vector, Burlingame, CA). In some experiments, slices were resectioned at 50 μ m on a vibratome before incubation with streptavidin-conjugated fluorescent dyes to reveal the complete axon arbors. For *in vivo* injections, two brains were cut sagittally and another two were cut transversally at 40-50 μ m on a vibratome after post-fixation. The sections were then treated with the fluorescent procedure.

Labeled cells revealed with the fluorescent dye were examined with a microscope equipped with epifluorescence and photographed with a confocal laser scanning microscope (Zeiss, LSM510, Germany). Images of labeling were captured at 25 $\times$ or 40 $\times$ oil objectives, with an aid of a zoom feature sometimes. A 543-nm laser was used to excite Alexa 594 or Texas Red (labeling) and emitted wavelengths between 601-719 nm were collected with a META detector. A 488-nm laser was used to excite Alexa 488 (Nissl staining) and emitted wavelengths were passed through a 500-550 nm bandpass filter before collection. Software LSM5 Image Browser (Zeiss) was used to store and manipulate the images. Our morphological identification of stained cells in the mormyrid valvula was based on

descriptions of Golgi stained material by Nieuwenhuys and Nicholson (1969a, b) and Meek (1998).

To visualize cells and fibers labeled with diaminobenzidine (DAB; Sigma, St. Louis, MO) as chromagen, slices were routinely resectioned at 50 μm . The endogenous peroxidase activity was bleached by a mixture of 10% methyl alcohol and 3% hydrogen peroxide in 0.1 M PB. Sections were incubated with an ABC kit (Vector; 1:200 in 0.1 PB) overnight, followed by a standard DAB procedure (Han et al., 1999; 2006). Sections were counterstained with Richardson's staining, dehydrated, cleared, and mounted in Eukitt.

Morphologies of the labeled cells and fibers were examined in an ordinary light microscope. Selected cells were examined under oil lens at 100 $\times$ and drawn on tracing paper from individual sections with aid of a camera lucida. Cells were reconstructed from these single drawings and inked, and digitalized with a scanner for final presentation. Statistical significance was determined with Student's t-test, and results are presented as mean $\pm$ S.E.M.

Results

Anatomy of the ridged valvula

The mormyrid valvula was originally described as a large sheet-like structure that covers the entire dorsal and lateral aspects of the entire brain, with the rostro-lateral parts folded up (Nieuwenhuys and Nicholson, 1969a; Bell and Szabo, 1986). A newer immunohistochemical analysis concluded that the mormyrid valvula consists of the sheet-like structure and lobe C1 (Meek, 1992), which was earlier described as part of the central lobes (Nieuwenhuys and Nicholson, 1969b; Bell and Szabo, 1986). The present study is concerned only with the sheet-like structure, or ridged valvula. The ridged valvula is made up of a layer of granule cells above which lies a series of parallel ridges. These ridges are a continuous ribbon-like tissue about 200 μm wide and 400 μm in height, which is folded back and forth upon itself and estimated to be more than 1 meter long (Fig. 1).

It should be clear that the orientation of the valvular ridges is different from that of the central lobes or whole brain. As illustrated in Figs. 2A and B, the transverse plane is a coronal section of a ridge (Fig. 2Ba) in which all five layers are always present and parallel fibers are preserved, as in the mammal. The sagittal sections are cut in parallel with the long axis of each ridge (Fig. 2Bb). In these sections two or three layers are visible, depending upon the cutting, and both parallel and climbing fibers are preserved at a right angle. In the horizontal sections cut parallel to the granular layer (Fig. 2Bc), four layers (2 ganglionic and 2 molecular) are seen dorsal to the granular layer, and climbing fibers and Purkinje cell dendrites are preserved, but parallel fibers are cut. Further details of this morphology are provided in the companion paper by Meek et al. (2007).

The histology of a valvular ridge is remarkably similar in any transverse section, consisting of two centrally located ganglionic layers with molecular layers on both sides, and all four layers lying above the granular layer base (Fig. 1B insertion and Fig. 2Ba). As illustrated in figure 2A, the molecular layer contains parallel fibers and the dendrites of Purkinje cells,

three groups of cells which were selected from the rostral, middle and caudal valvula respectively. The results showed that dendritic tree widths were $208 \pm 27 \mu\text{m}$ in the rostral group ($n=15$), $98 \pm 11 \mu\text{m}$ in the middle group ($n=9$), and $56 \pm 10 \mu\text{m}$ in the caudal group ($n=11$). The differences among three groups are statistically significant ($P<0.05$). Examples from each group and their proximal sampling locations are shown in Fig. 5.

Initially, attempts were made to reveal Purkinje cell axons in transverse slices, based on earlier anatomical studies which suggested that these axons should traverse down vertically to the basal area and there make synaptic contact with basal efferent cells, as illustrated in Fig. 2A. These attempts failed, however, despite the fact that somas and dendrites of Purkinje cells were consistently well-labeled in the same slices. Searches for the Purkinje cell axons were then shifted to horizontal slices, and resections were performed routinely for histology. Then, the axon arbors were revealed. These mostly arise from the lower side of the Purkinje cell somas (occasionally from one of the primary dendrites), become thin quickly, and then extend and give off branches with en passant and terminal buttons in the ganglionic layer that are best observed in the horizontal plane. While in most cases an axon arbor projects to one side of the soma without a preferred direction (Figs. 3A, 4A and B), in a few cases it extends to both sides (Fig. 5A). Compared to Purkinje cell axon arbors in the central lobes (Han et al., 2006) and caudal lobes (Campbell et al., 2007; Zhang and Han, 2007), axon arbors of valvular Purkinje cells are usually shorter and less branched, and thus more localized. Many Purkinje cells have axon arbors that do not extend beyond the width of their dendritic trees. In some cases, however, the axon arbors extended for up to 100-200 μm , twice as far as their dendrites. Examples of such cells are shown in Fig. 4. Thus, our results indicate that the axon arbors of valvular Purkinje cells do not project vertically down to the basal cell area of the ridges, as expected, but instead are oriented in the horizontal plane.

Physiologically, all valvular Purkinje cells showed characteristic physiological properties. The detailed physiology and pharmacology of Purkinje cells as well as that of other cell types in the valvula will be reported separately (Han et al., in preparation). Briefly, Purkinje cells responded to somatic voltage steps with two types of spikes, a small narrow spike and a large broad spike, as shown in Fig. 3C. Purkinje cells responded to parallel fiber stimulation with graded excitatory postsynaptic currents (EPSCs), that showed paired-pulse facilitation (Fig. 3D), and to climbing fiber stimulation with characteristic large all-or-none EPSCs, that showed paired-pulse depression (Fig. 3E). These physiological properties of valvular Purkinje cells are similar to those of central lobe Purkinje cells (Han and Bell, 2003), but differ from those of mammalian Purkinje cells in that the valvular Purkinje cells fire multiple types of spikes (Ito, 1984; Eccles et al., 1967). In many cases, inhibitory postsynaptic potentials (IPSPs) and currents (IPSCs) were observed when parallel fiber stimulation was applied (not shown).

Basal efferent cells

Basal efferent cells (basal cells) were identified as the major cell type that conveys information from the valvular ridges to other regions of the brain (Nieuwenhuys and Nicholson, 1969b; Finger et al., 1981). They can be recognized in living slice preparation by

their large somas and restrictive localization, with their cell bodies usually embedded in a fiber mesh. Typically, only 1-3 basal cells per ridge were seen in transverse slices, and a line of such cells was observed at intervals of a few to over 10 μm in horizontal slices. A total of 77 basal cells were recorded, and 42 of them were morphologically identified.

The cell bodies of basal cells are usually rounded or multipolar in shape and larger in size than Purkinje cells (with 15-20 μm and ~ 10 μm diameters, respectively). Two to four primary dendrites usually arise from the molecular side of the soma, radiate out in the ganglionic layer, and extend to the molecular layer where they branch further. Because of the location of their somas in the ganglionic layer, basal cell dendritic trees are asymmetrically oriented in the transverse plane (Fig. 6B), but are symmetrically oriented in the horizontal plane (Fig. 6A). In both planes, the dendritic trees extend a similar range of 150-200 μm in the molecular layer and no branches have been observed in the granular layer. Basal cell dendrites are usually thin and smooth, and in many cases carry swellings (Fig. 9C-E). Although less regular than Purkinje cell dendrites, those of the basal efferent cells traverse somewhat parallel to each other and orthogonally to the parallel fibers in the molecular layer.

Basal cell axons were normally cut when the cells were labeled in the transverse plane (Fig. 8A), but could be followed for long distances when the cells were labeled in the sagittal plane (Fig. 8B) or the horizontal plane (Fig. 8C). These axons are typically thick, presumably myelinated, and have no collaterals.

Basal efferent cells are also distinct from Purkinje cells in their physiology, firing a single type of large narrow spikes in response to somatic current injections (Fig. 6C). Parallel fiber stimulation evoked graded EPSPs with minimal paired-pulse facilitation (Fig. 6D). In many cases, IPSPs or IPSCs were also observed when parallel fiber stimulation was applied (not shown). No large all-or-none response was obtained when stimulation electrodes were repositioned around the recorded cell bodies, indicating that these cells receive parallel fiber input but not climbing fiber input, as is characteristic of Purkinje cells in the central lobe (Han and Bell, 2003).

Large central cells

As early Golgi studies suggested that valvular efferent cells were not restricted to the basal regions of the ridges, some large cells in the ganglionic layer above the basal area have also been classified as efferent cells, namely vertical cells and small central cells (Nieuwenhuys and Nicholson, 1969b). We reexamined these cells in slice preparations, with a total of 46 non-Purkinje cells recorded outside the basal area and 34 of them morphologically identified. These cells, however, fell into at least two subtypes morphologically. The vast majority of them we named *large central cells* ($n=27$), while others belonged to the vertical and small central cell groups (see below).

The cell bodies of large central cells were similar to those of basal efferent cells as described above; rounded and multipolar in shape and ~ 15 μm in diameter. All large central cells were located in the ganglionic layer above the basal area. Their dendritic trees were largely similar to those of the basal efferent cells, extending for 100-200 μm but appearing to be symmetric in both the transverse and horizontal planes (unlike the dendritic trees of basal

cells, which are symmetric only in the horizontal plane). Large central cell dendrites are thin and smooth, and line up somewhat parallel with each other, perpendicular to the parallel fibers in the molecular layer. Many dendrites carried swellings even in the cases when recordings ended with the cells in good condition. The most characteristic morphological feature of large central cells is their axon pattern, which includes a thick process arising from basal side of the soma opposite dendrites that can be followed for a long distance in the transverse plane, indicating a myelinated long projection. The main axon typically has one or several thin branches that may further branch and terminate in the ganglionic layer with en passant and terminal buttons, as illustrated in Figs. 7A and B. The two large central cells shown were reconstructed from horizontal sections. In both cells, the main axon and its collaterals were confined to the ganglionic layer, but extended far beyond the range of the dendritic tree. A photomicrograph from a 200 μm horizontal slice of another large central cell with similar morphology is shown in Fig. 8D. In the transverse plane, however, the main axons of larger central cells were usually cut and the axon collaterals were minimal, while the dendritic trees were similar to those seen in the horizontal section of Fig. 8E.

The physiological properties of large central cells were largely uniform and similar to those of basal efferent cells, e.g., firing repetitively with a single type of large narrow spikes in response to somatic current injection, and responding to parallel fiber stimulation with a graded EPSCs that showed moderate paired-pulse facilitation (Fig. 7C).

Small central cells

Three cells in our sample were identified as *small central cells* and resembled the central cells as described by Nieuwenhuys and Nicholson (1969b) closely. The best example is shown in figure 10A. The cell bodies of small central cells are located in the ganglionic layer, and their dendrites are largely distributed in the ganglionic layers as well. We were uncertain whether their dendrites also extended into the molecular layer, but some of their dendrites clearly extended into the granular layer. Similar to large central cells, the axons of central cells are rather thick and have several thin collaterals.

Small central cells fired a single type of large narrow spikes to somatic current injection (Fig. 10B), and responded to parallel fiber stimulation with EPSPs or EPSCs.

Vertical cells

Four cells were classified as *vertical cells* based primarily on their dendritic trees, as described by Nieuwenhuys and Nicholson (1969b). The cell bodies of these cells are slightly larger than those of Purkinje cells and are located in the ganglionic layer, distal to the basal area. Two main thick dendrites arise from opposite poles of the soma and extend to opposite (basal and distal) directions in the ganglionic layer. The cell bodies also give off several thinner dendrites towards the molecular layer. The two main dendrites typically give off branches repeatedly at nearly regular intervals, which course somewhat parallel to each other in the molecular layer. The thinner primary dendrites also send branches to the molecular layer in the same plane as two main dendrites. Thus, vertical cells have roughly planar, vertically oriented dendritic trees that cover much of the molecular layer in the transverse plane (Fig. 10C). Similar to large central cells, the axons of vertical cells include a thick

main branch and several thin collaterals. However, the axon arborizations of vertical cells are oriented vertically, similar to their dendritic trees, and thus different from large central cells.

Physiologically, vertical cells were indistinguishable from small central cells.

Golgi cells

In mammalian cerebellum, Golgi cells receive parallel fiber input by their molecular layer dendrites and inhibit granule cells by their granular layer axons (Ito, 1984). However, this cell type has not been fully examined in mormyrid cerebellum. In the present study, 9 cells were morphologically classified as *Golgi cells*, all of them sampled along the border between the granular layer and the molecular/ganglionic layers. Golgi cells have large multipolar somas (~15 μm in diameter). Several primary dendrites usually arise from molecular side of the soma and branch two or three times irregularly. The final dendrites are fine and smooth, and traverse parallel to each other in the molecular layer toward the surface of the ridge. Typically one thick axon arises from the soma and projects down to the granular layer, where it branches repeatedly and irregularly and ends with en passant and terminal buttons. One of such Golgi cells (n=4) is shown in Fig. 11A, its axon branched extensively and all its terminal arbors confined to the granular layer.

Axon arbors of some Golgi cells, however, were not confined in the granular layer. As axons of these cells extended to both the granular layer and the ganglionic layer, these cells were named *Golgi-like cells* (n=5). It is clear that other morphological features of Golgi-like cells are very similar to those of Golgi cells. An example of a Golgi-like cell is shown in Fig. 12.

Physiologically, both subtypes of Golgi cells were undistinguishable, firing a single type of large narrow spikes repetitively in response to somatic voltage or current steps, and responding to parallel fiber activation with graded EPSCs or EPSPs (Figs. 11B and 12B), with minimal paired-pulse facilitation.

Deep stellate cells

Stellate cells are small inhibitory interneurons found scattered in the molecular layers of mammalian cerebellum (Ito, 1984) and mormyrid cerebellum (Meek and Nieuwenhuys, 1991; Campbell et al., 2007). Material stained with antibodies against calcium binding proteins calretinin and calbindin showed, however, that a great number of stellate cells are also distributed in the upper ganglionic layer of the mormyrid valvula. These cells were named *deep stellate cells* (Meek et al., 2007). To know more about their morphology, special effort was made to examine them in slice preparations. We were able to record from 23 small cells in the ganglionic layer which showed similar physiological properties and 11 of these were successfully labeled and identified as deep stellate cells.

Morphologically, the deep stellate cells have small cell bodies (6-8 μm in diameter), located between the molecular and ganglionic layers. The dendrites are usually fine and smooth, and are sometimes indistinguishable from their axon collaterals in the molecular layer. The dendritic trees are confined to the molecular layer and appear to be planar and are largely oriented in the horizontal plane, where their 100-200 μm span is similar to that of Purkinje cells. Figure 13A shows the labeling of one deep stellate cell (arrow). Two more cell bodies

with minimal processes were also labeled in this preparation, probably by dye coupling. This image was taken from a horizontal section of 40 μm . Images of adjacent sections also showed labeling with a similar pattern but with many fewer processes, suggesting a complete labeling of the cell. Deep stellate cell axons usually arise from the soma and extend in both the ganglionic layer and the lower molecular layer, with en passant and terminal buttons. Another example is shown in Fig. 13B. This image was taken from a transverse slice of 80 μm (optical thickness), in which a few fine dendrites are labeled in the molecular layer while the axon arbor traverses more than 300 μm toward the base of the ridge in the ganglionic layer.

Physiologically, deep stellate cells differ from all other cell types described above by showing rapid spike frequency adaptation to somatic current injection (Fig. 13C, top). Deep stellate cells also respond to parallel fiber stimulation with graded EPSCs or EPSPs that show minimal paired-pulse facilitation (Fig. 13C, bottom).

Parallel fibers

Fibers and cells were labeled when tracer was injected in the molecular layer, while only fibers were labeled when tracer was injected in the granular layer. We used slice preparations similar to those for single-cell recording and labeling, except that the slice thickness was increased from 200 μm for single-cell labeling to 350 μm for local injection. In transverse sections, molecular layer injection resulted in retrograde labeling of small cells in the granular layer, as well as labelings of medium and large cells near the injection sites. Interestingly, the labeled small cells were exclusively confined to the granular region directly beneath the molecular layer where the tracer was injected, as shown in Fig. 14A. Furthermore, a large number of small cells were found to project their axons to form a tight fiber bundle that traversed through the molecular layer (Figs. 14B and C). Axons of these small cells do not bifurcate, but instead run parallel to each other in the molecular layer towards the apex of the valvular ridges. The fibers are usually fine and straight, with en passant buttons at intervals of 3-10 μm (Fig. 14D), and appear similar to the parallel fibers described by Nieuwenhuys and Nicholson (1969b). The retrogradely-labeled small cells in the granular layer usually have small cell bodies (4-5 μm in diameter), with three or four short dendritic branches, each terminating in a small knob (Fig. 14E). The morphology of these cells identifies them as the granule cells as described by Nieuwenhuys and Nicholson (1969b). When injections were made to granular layer, in addition to the anterogradely-labeled parallel fibers described above, fibers of a variety of diameters were also observed in the granular layer. These fibers are similar to the mossy fibers described in mammalian cerebellum (Ito, 1984). They do not have a clear orientation, but instead randomly traverse and branch, and end with variously-sized en passant and terminal buttons (Fig. 14F).

Climbing fibers

Anterograde transport of BDA from the inferior olive resulted in clear labeling of fibers in the valvular ridges as well as in other cerebellar structures. In both transverse and horizontal sections, labeling was confined to the ganglionic layer of a certain portion of the valvula in all cases, suggesting incomplete labeling of the pathway and a topographic organization of the projection. In the transverse plane, fibers with variety of diameters were observed in one

side of the ganglionic layer (Fig. 15A). The thick fibers were distributed in the lower ganglionic layer and were always cut, with the cut ends often seen at the top and bottom surfaces of the sections. The fine fibers with en passant and terminal buttons were usually distributed in the upper ganglionic layer. In the horizontal plane, however, the thick fibers could be followed for over several millimeters (Fig. 15C). Fine fibers with terminal buttons could also be seen (Fig. 15B). These results indicate that the valvular ridges receive climbing fibers from the inferior olive nuclei, as in the mammal (Ito, 1984). The termination patterns of climbing fibers between the two cerebellums, however, are clearly different. In the valvula cerebelli, climbing fibers terminate exclusively in the ganglionic layer and do not invade the molecular layer, whereas in mammalian cerebellum, climbing fibers terminate nearly exclusively in the molecular layer (Ito, 1984). Furthermore, valvular climbing fibers are largely oriented in the horizontal plane, as are the Purkinje cell dendrites, similar to the central lobes (Meek and Nieuwenhuys, 1991; Han et al., 2006).

Discussion

The morphology of the mormyrid valvula has been previously examined using Nissl and Golgi methods (Nieuwenhuys and Nicholson, 1969a, b), electron microscopy (Kaisermann-Abramhof and Palay, 1969), tract-tracing (Finger et al., 1981), and recently immunohistochemistry (Meek et al., 2007). The present study complements these studies using intracellular and extracellular labeling and recording techniques. The main new findings of present study are: 1) the horizontal orientation of Purkinje cell axon arbors; 2) the newly identified large central cells having long, myelinated axons with local collaterals; 3) the extensive axon arbors of Golgi cells in both the granular and ganglionic layers; 4) identification of deep stellate cells; and 5) the restriction of climbing fibers to the ganglionic layer in the horizontal plane. The local circuitry of the valvular ridges, based on current findings as well as results from previous studies, is summarized in Fig. 16, in which all essential morphological features of the valvular ridges are shown.

Striking aspects of the valvular circuitry are the palisade pattern of the Purkinje dendrites, the horizontal orientation of the dendritic trees as well as axon arbors of Purkinje cells, the presence of large basal efferent cells in the proximal part of the ridges and the presence of large central cells more distally, the extensive axon arborizations of Golgi-like cells in both the granular and ganglionic layers, the unbranched origin of parallel fibers from granule cell axons and the restriction of climbing fiber terminations to the proximal dendrites and cell bodies of Purkinje cells in the ganglionic layer. Some of these features are also present in other cerebellar structures of mormyrid. For example, spatial segregation of parallel fiber and climbing fiber inputs and the palisade pattern of Purkinje cell dendrites are also observed in the central lobes (Nieuwenhuys and Nicholson, 1969b; Meek and Nieuwenhuys, 1991; Han et al., 2006; Meek et al., 2007), while the proximity of Purkinje cells to their target cells is also present in the central lobes (Nieuwenhuys and Nicholson, 1969b; Meek and Nieuwenhuys, 1991; Han et al., 2006; Meek et al., 2007) and the caudal lobes (Zhang and Han, 2007; Zhang et al., 2007). These aspects have been discussed in detail (Han and Bell, 2003; Han et al., 2006; Meek et al., 2007). Some features, however, exist only in the valvula, such as the absence of ascending segments of granule cell axons, restrictively located basal efferent cells and the presence of the newly-identified inhibitory deep stellate

cells (Meek et al., 2007). Here, our discussion will be focused on some significant new findings and unique features that exist only in the valvula ridges.

Cerebellar circuitry in the valvular ridges

Previous work using the Golgi method showed that the palisade dendrites of Purkinje cells are oriented in the horizontal plane, perpendicular to the vertically-oriented parallel fibers. Axons of Purkinje cells were presumed to descend to the basal area and make synaptic contacts with basal efferent cells, as illustrated in Fig. 2A (Nieuwenhuys and Nicholson, 1969b), the latter in turn conveying information from the valvular ridges to other regions of the brain. However, our results show that Purkinje cell axons do not descend to the base of the ridges, but rather project horizontally and terminate in the same plane as their dendritic trees. This is consistent with our recent immunohistochemical analysis showing that Purkinje cells in the distal portion of the valvular ridges do not project down to the basal area of the valvular ridges. Instead, the basal efferent cells in this region receive heavy input from deep stellate cells (Meek et al., 2007). Thus, the identity of target cells of the Purkinje cells in the distal portions of the valvular ridges remains unclear. It is certainly possible that Purkinje cells innervate neighboring Purkinje cells, as has been confirmed by our recent dual cell recordings in the mormyrid caudal lobes and central lobes (unpublished observations) and by earlier morphological study in the mammal (Bishop and O'donoghue, 1986). It is equally possible and more likely that Purkinje cells innervate efferent cells at same horizontal level of the ridge, where the efferent cell axons may join the basal bundle as Nieuwenhuys and Nicholson originally proposed (1969b). It is not clear at this stage which cell type(s) identified in the present study, e.g., large central cells, small central cells or vertical cells, are the best candidates for Purkinje cell targets, but it is also clear that efferent cells are present at all levels of the ridges (see next section). This issue could be addressed in future using different approaches, such as dual cell recordings and combining retrogradely labeling of efferent cells with immunostaining of Purkinje cell axons that are positive to Zebrin II.

The nucleus lateralis is the main target of valvular efferent cells (Finger et al., 1981). In intact animals, the retrograde transport of BDA from the nucleus lateralis labeled cells that were distributed in the basal region and at all levels along the ganglionic layer of the valvular ridges (our unpublished observations). It would be difficult to identify which of the cell types filled in this study were indeed efferent cells, as dendritic arbors of retrogradely labeled cells are usually incomplete. Nevertheless, it is clear that efferent cells are indeed present along the ganglionic layer above the basal region of the ridges. The vertical cells and small central cells described in earlier Golgi studies (Nieuwenhuys and Nicholson, 1969b) and further characterized here are certainly the best efferent cell candidates.

The large central cell newly identified in the present study may potentially also convey information from valvular Purkinje cells to other regions of the brain because its dendrites are well within the range of Purkinje cell axons, while its myelinated axon projects long distances in the horizontal plane. However, evidence of large central cell axons joining the basal bundles is still lacking. It is possible that the large central cells send their long myelinated axons to other parts of the valvular ridges as commissural neurons, as do the

large multipolar interneurons (LMI) of the mormyrid ELL (Meek et al., 2001), rather than projecting outside the cerebellum as do true efferent cells. Therefore, the distal target of large central cells requires further investigation.

The deep stellate cell is a unique cell type that is present only in the valvula ridges and lobe C1 (Meek et al., 2008). Several lines of evidence suggest that deep stellate cells may play an important functional role in the valvular ridge circuitry. First, they are abundant and greatly outnumber the conventional stellate cells that are scattered in the molecular layer. Second, they are inhibitory in nature. Materials stained with antibodies against calretinin and calbindin as well as GABA have shown that axon terminals of deep stellate cells heavily appose on basal efferent cells and, to a lesser degree, on Purkinje cells and other efferent cell types (Meek et al., 2008). We have demonstrated that deep stellate cells receive parallel fiber input by way of their molecular layer dendrites. All these results suggest that deep stellate cells are well suited to generate a strong feedforward inhibition on both efferent cells and Purkinje cells in the valvular ridges. This hypothesis is consistent with our recent electrophysiological observations in valvular slice preparations. Strong synaptic inhibition was recorded from both basal efferent cells and Purkinje cells when parallel fibers were activated by molecular layer stimulation, and pharmacological tests showed that this inhibition was mediated by GABA_A receptors. With inhibition pharmacologically blocked, synaptic excitation of these two cell types was dramatically enhanced (Zhang et al., 2006). The hypothesis of a feedforward inhibition generated by deep stellate cells was further strengthened by our most recent results of dual cell recording, in which single or burst firing of a deep stellate cell induced an inhibitory synaptic potential in a Purkinje cell (Han et al., in preparation).

Another unique morphological feature of the valvular circuitry, as illustrated in Fig. 16, is that granule cell axons lack a so-called ascending segment (in mammalian cerebellum, the portion of the granule cell axon proximal to its bifurcation.) In the valvular ridges, instead of bifurcating the granule cell axons ascend to the molecular layer as parallel fibers. This is important since ascending segments were proposed as playing an important role in signal transmission from granule cells to Purkinje cells, based on anatomical observations that more synaptic contacts can be made between ascending segments and a given Purkinje cell than between parallel segments and a given Purkinje cell (Gundappa-Sulur et al., 1999; Napper and Harvey, 1988). The two segments of the granule cell axon appear to differ physiologically as well (Sims and Hartell, 2005). It would be important to know the physiological role(s) played by the different portions of the granule cell axon in the cerebellar circuitry. To our knowledge, the best way to address this issue experimentally would be to examine and compare synaptic transmission to Purkinje cells from granule cells with and without ascending segments. The valvular ridge seems to be an exemplary model for the investigation of this issue.

Functional considerations

As pointed out by Meek and Nieuwenhuys (1991), Meek (1992a, b) and Meek et al. (2008), the palisade pattern of mormyrid Purkinje cell dendrites is a specialization exclusively involved in the processing of parallel fiber input, since climbing fibers do not enter the

molecular layer. Apparently, the mormyrid central lobes may have optimized and extended parallel fiber signal processing to its extreme capacity by extending the general orthogonal organization of the molecular layer to a third dimension. Not only are the parallel fibers orthogonal to the horizontally-oriented spiny dendritic trees of Purkinje cells but, in addition, the spiny dendrites are oriented parallel to each other in the horizontal plane and remain orthogonal to the sagittal and transverse directions, both of which are perpendicular to the meningeal surface, as illustrated in Fig. 2A. The most regular palisade pattern (i.e. with the fewest branching points of spiny dendrites) is encountered in the valvular ridges, where ascending segments of granule cell axons are absent (Meek and Nieuwenhuys, 1991). In the lobus transitorius, parallel fibers originate from laterally-located granule cell masses and thus enter the molecular layer from both sides, without a T-bifurcation (Meek, 1992b). In the ridged valvula that accounts for most of the valvular cell mass, parallel fibers enter the molecular layer from only one side (Fig. 2A), again without a T-bifurcation. So, the mormyrid valvula seems an ideal cerebellar specialization in which to investigate the functional significance of the orthogonal organization of the cerebellar molecular layer. In the valvula, different aspects of this organization can be studied independently of climbing fiber effects. It may be expected that the results will also yield insight as to the significance of the less distinct, but still remarkable two-dimensional orthogonal organization of the molecular layer of other vertebrates, including mammals.

The only hypothesis presently available to explain the functional significance of the orthogonal organization of the cerebellar molecular layer, both in mammals and mormyrids, is that Purkinje cells function as coincidence detectors of parallel fiber input (Meek, 1992b). If that is indeed the case, Purkinje cells would subserve the spatial coding of temporal differences in mossy fiber inputs, with each being optimally tuned to the specific mossy fiber input pattern that results in the maximal coincidence of parallel fiber input at that Purkinje cell's dendritic tree (Meek 1992b). In the configuration of mammalian cerebellum, this time coding has been proposed to result in the detection of specific "tidal waves" of mossy fiber input patterns in the transverse direction (Braitenberg, 1967; Braitenberg et al., 1997). In the mormyrid lobus transitorius, it may result in the detection of specific time differences between mossy fiber inputs to the left and right granule cell populations (Meek, 1992b), while in the ridged valvula it might be used to detect specific mossy fiber input waves originating from granule cells at deep to superficial levels in the basal plate of the granular layer (Meek et al., 2008).

In addition to the valvular specializations surveyed above, it should be noted that the location and organization of the efferent cerebellar neurons in teleosts can also be considered as a parallel fiber specialization. These efferent cells are located in the ganglionic layer and have non-spiny dendrites in the molecular layer. Thus, they do not receive mossy fiber input, as deep cerebellar nuclei efferent neurons in mammals do, but instead receive massive parallel fiber input (Meek et al., 2007). Moreover, these mormyrid efferent cells do not receive climbing fiber input (Meek et al., 2007), whereas the deep cerebellar nuclei in mammals receive a collateral innervation from the olivocerebellar climbing fibers (Ito, 1984). Also in contrast to the cerebellums of mammals and other vertebrates, the organization of both cerebellar efferent neurons and Purkinje cells in the mormyrid valvula

seems to optimize the processing of specific parallel fiber input patterns at the expense of mossy fiber as well as climbing fiber input patterns.

In summary, the local circuitry of the ridged valvula has been characterized here as well as in a recent immunohistochemical study (Meek et al., 2008). The great similarities between the mormyrid cerebellum and mammalian cerebellum (Han and Bell, 2003; Han et al., 2006) suggest that what is learned about mormyrid cerebellum will shed light on the many open questions regarding cerebellar function in mammals. The differences between the two cerebellums are, in fact, unique features of mormyrid cerebellum that may provide opportunities for investigating important aspects of cerebellar function which are not possible to address in the mammalian model.

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Fig. 1. Anatomical overview of mormyrid cerebellum

A. Dorsal view of the brain, showing that the entire brain is covered by the valvula. The smooth portions are the bottom of the valvular sheet folded up. The medio-lateral strips are the tops of the valvular ridges.

B. Dorsal view of the brain with the valvula unfolded. Insertion shows transverse view of ridges.

C. Sagittal view of the brain. All structures above the dashed line are cerebellum or cerebellar-like structures.

Abbreviations: C1-4, central lobes C1-4 of the cerebellum; Cr, cerebellar crest; CLa, anterior caudal lobe; CLp, posterior caudal lobe; ELL, electrosensory lobe; ggl, ganglionic layer; gran, granule layer; LT, lobus transitorius of the cerebellum; mol, molecular layer; Va, valvula of the cerebellum. All figures are adapted with permission from Nieuwenhuys and Nicholson (1969a). Scale bars = 2 mm in A, B; = 1 mm in C.

Fig. 2. Organization of the valvular ridge

A. A schematic drawing of a valvular ridge, showing organization of cells and fibers in three dimensions. Insertion shows presumed connections among the different cell types. Figure is adapted with permission from Nieuwenhuys and Nicholson (1969a).

B. Schematic drawings, showing the laminar organization of a valvular ridge in different planes, as defined in this study: a, transverse plane; b, sagittal plane; c, horizontal plane.

C. Experimental arrangements. Schematic drawing of a Purkinje cell viewed in the horizontal plane. Purkinje cell axon is shown in blue, parallel fibers in green and climbing fiber in red. Stimulus electrodes are placed in the molecular layer (SM) and ganglionic layer (SG) to activate parallel fibers and climbing fibers, respectively.

Abbreviations: B, basal cell; bb, basal bundle; cf, climbing fiber; dst, deep stellate cell; G, Golgi cell; ggl, ganglionic layer; gr, granule cell; gran, granular layer; mol, molecular layer; mf, mossy fiber; P, Purkinje cell; pf, parallel fiber; Rec, recording electrode; sc, stellate cell (molecular layer); SG, stimulation of ganglionic layer; SM, stimulation of molecular layer; vc, vertical cell. Scale bar = 50 μ m in C.

Fig. 3. Morphology and physiology of Purkinje cells

A and B. Reconstructions of two Purkinje cells from horizontal (A) and transverse (B) sections, respectively. Note that the dendritic trees and axon arbors of Purkinje cells are complete in the horizontal plane but both were cut in the transverse plane. Also note that in this figure, as well as in the following figures, reconstructions were made from thin sections (40-50 μm thickness) after a DAB procedure. Insertions show the orientations of cells in the ridges.

C. Typical responses of a Purkinje cell to a somatic voltage step (single trace): small narrow spikes and large broad spikes.

D. Averaged EPSC response of a Purkinje cell to molecular layer stimulation (SM), showing paired-pulse facilitation.

E. A Purkinje cell responds to ganglionic layer stimulation (SG) with large all-or-none EPSCs. The responses (overlay of single traces) show clear paired-pulse depression (ppd, black traces). The stimulus intensity was near the threshold, and if the first stimulus failed, the response to second stimulus was near the full size (gray trace). Abbreviations are the same as in Fig. 2. Scale bars = 10 μm in A, B.

Fig. 4. Photomicrographs of Purkinje cells labeled with fluorescent dye

A. A Purkinje cell labeled in the horizontal plane. The soma, proximal dendrites and axon (ax) are confined to the ganglionic layer (ggl), and the palisade dendrites are confined to the molecular layer (mol). Note in this figure and following figures that fluorescent labeled cells were processed and photographed from 200 μ m slices unless otherwise noted.

B. Two Purkinje cells were labeled in a horizontal slice, with axons projecting in opposite directions in the ganglionic layer.

C. A Purkinje cell labeled in a transverse plane. The dendritic tree is narrow and the axon was cut (arrow) during slicing. Nissl counterstaining is shown in green in this and the following figures. Abbreviations are the same as in Fig. 2. Scale bars = 20 μ m in A, B; 50 μ m in C. See magenta-green version of this figure in supplementary files.

Fig. 5. Comparisons of dendritic tree sizes of Purkinje cells from different regions of the valvula
 A-C. Representative cells reconstructed from horizontal sections, from the rostral (A), the middle (B) and the caudal (C) portions of the valvula, respectively.
 D. The proximal valvular regions from which the three groups of cells were sampled.
 Abbreviations are the same as in Fig. 2. Scale bars = 10 μ m in A-C.

Fig. 6. Morphology and physiology of basal efferent cells

A and B. Reconstructions of two basal efferent cells from horizontal (A) and transverse (B) sections, respectively. The axons of both cells are thick and have no collaterals. Insertions show cell orientations in the ridges. Note the symmetric (A) and asymmetric (B) dendritic trees.

C. Response (single trace) of a basal efferent cell to a somatic current step of 200 pA.

D. Averaged responses of a basal efferent cell to stimulation of molecular layer (SM). The EPSPs show a minimal paired-pulse potentiation. Abbreviations are the same as in Fig. 2. Scale bars = 10 μ m in A, B.

Fig. 7. Morphology and physiology of large central cells

A and B. Reconstructions of two large central cells in horizontal planes. Both cells have a thick axon that traverses long distances in this plane and gives off local collaterals which are largely confined to the ganglionic layer.

C. Responses (single trace) of a large central cell to a somatic current step of 200 pA (top), and averaged responses to stimulation of molecular layer (SM, bottom). The EPSCs show moderate paired-pulse facilitation under voltage clamp. Abbreviations are the same as in Fig. 2. Scale bars = 50 μ m in A, = 40 μ m in B.

Fig. 8. Photomicrograph of efferent cells labeled with fluorescent dye. Nissl counterstaining showed in green

A. A basal efferent cell labeled in a transverse slice, with a cut thick axon (ax).
 B. A basal efferent cell labeled in a sagittal slice, with a long thick axon (ax) in the granular layer.
 C. A basal efferent cell labeled in a horizontal slice, with a long thick in the ganglionic layer.
 D. A large central cell labeled in a horizontal slice, with a long thick axon and thin local collaterals in the ganglionic layer.
 E. A large central cell labeled in a transverse slice, with a cut thick axon and minimal local collaterals in the ganglionic layer. Abbreviations are the same as in Fig. 2. Scale bars = 50 μ m in A, B, D; =25 μ m in C, E. See magenta-green version of this figure in supplementary files.

Fig. 9. Photomicrographs of dendritic arbors of different cell types. Cells were stained with DAB as chromagen in thin sections

A and B. A Purkinje cell, showing the typical palisade dendritic tree in the molecular layer (A) and spiny dendrites at higher magnification (B).

C and D. A basal efferent cell, showing swellings along the dendrites in the molecular layer.

E. A basal efferent cell, showing smooth dendritic arbors. Scale bars = 20 μm in A, C; 10 μm in B, D, E.

Fig. 10. Photomicrographs of a vertical cell and a small central cell labeled with fluorescent dye
 A. A vertical cell labeled in a slice that was cut obliquely. Its dendrites occupy much of the molecular layer (mol) of the ridge, and its axon arbors are largely confined to the ganglionic layer (ggl).
 B. A small central cell labeled in a sagittal slice. Its dendrites are largely confined to the ganglionic layer (ggl) where its thick axon with local collaterals are also located.
 C. Responses of a vertical cell (same cell as shown in A) to somatic current injection.
 Abbreviations are the same as in Fig. 2. Scale bars = 20 μm in A, B. See magenta-green version of this figure in supplementary files.

Fig. 11. Morphology and physiology of a Golgi cell

A. A Golgi cell labeled with fluorescent dye in a sagittal slice, as shown schematically in the insertion. This cell has a large multipolar soma in the ganglionic layer (ggl). Its smooth and non-planar dendritic tree is confined to the molecular layer (mol), while its axon branches extensively in the granular layer (gran).

B. A Golgi cell (same cell as shown in A) fired a single type of spikes in response to a somatic current injection of 200 pA (left, single trace), and responded to stimulation of the molecular layer (SM) with EPSCs under voltage clamp (right, average). The responses showed a moderate paired-pulse facilitation. Abbreviations are the same as in Fig. 2. Scale bars = 40 μ m in A. See magenta-green version of this figure in supplementary files.

Fig. 12. Morphology and physiology of a Golgi-like cell

A. A Golgi-like cell labeled with fluorescent dye in a transverse slice, as shown schematically in the insertion. The characteristic morphological feature of this cell is the way its axon arbors project into both the granular layer (gran) and the ganglionic layer (ggl). B. Similar to Golgi cell shown in Fig. 11, this Golgi-like cell fired a single type of spikes with adaptation in response to a somatic current injection of 200 pA (left), and responded to stimulation of molecular layer (SM) with an EPSP under current clamp (right).

Abbreviations are the same as in Fig. 2. Scale bars = 20 μ m in A. See magenta-green version of this figure in supplementary files.

Fig. 13. Morphology and physiology of deep stellate cells. Cells were labeled with fluorescent dye and histology was carried out in resectioned sections (A) or whole slice (B)

A. A deep stellate cell was photographed from horizontal sections of 40 μm . Its small soma (arrow) was located in the upper ganglionic layer, and the fine dendrites and axon arbors are hardly distinguishable. Note that one cell was recorded and injected, and two small cells nearby were also labeled, with minimal processes.

B. Another deep stellate cell was labeled and photographed from a transverse slice of 200 μm . Its dendrites were clearly cut and axon arbors were confined to the ganglionic layer (gran).

C. Recording from a deep stellate cell (same cell as shown in A) under current clamp shows a rapid adaptation of spikes evoked by somatic current step (top), and minimal paired-pulse facilitation of parallel fiber EPSCs (bottom). Abbreviations are the same as in Fig. 2. Scale bars = 20 μm in A, =50 μm B. See magenta-green version of this figure in supplementary files.

Fig. 14. Photomicrographs of labelings in slice preparation. Tracer was injected in transverse slice and labelings were revealed with red fluorescent dye after resection. Nissl counterstaining is shown in green

- A. Injection site was located in the molecular layer (mol), and all retrogradely labeled granule cells were confined to the granular layer (gran) directly beneath the molecular layer where the injection site was located (arrow).
- B. Another molecular layer injection that shows all axons of labeled granule cells as combining into a small bundle (arrow) before entering the molecular layer (mol). Note that at least one Purkinje cell (arrowhead) was labeled with cut dendrites in this plane.
- C. An adjacent section to the one showed in B, showing the same parallel fiber bundle running in the proximal molecular layer (mol), above the ganglionic layer (ggl). Note at least one Purkinje cell (P) with cut flat dendrites and a basal efferent cell (B) with broad dendrites in the basal region of the ridge.
- D. An enlarged image, showing single parallel fibers with en passant buttons in the molecular layer (mol).
- E. Retrogradely labeled granule cells, with short dendrites that end with knobs.
- F. Mossy fibers labeled by injection of tracer into the granular layer (gran). Note the branching of the thick fibers and the large en passant buttons of the thin fibers.
- Abbreviations are the same as in Fig. 2. Scale bars = 100 μ m in A, = 40 μ m in B; = 50 μ m in

C; = 20 μm in D; = 10 μm in E; = 24 μm in F. See magenta-green version of this figure in supplementary files.

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Fig. 15. Photomicrographs of climbing fibers in intact animals. Tracer was injected to the inferior olive and labeling was revealed with red fluorescent dye. Nissl counterstaining is shown in green

- A. Transverse view, showing that labeled climbing fibers are confined in the ganglionic layer (ggl). The thick fibers were usually cut and thin fibers have en passant and terminal buttons. Note climbing fibers are only present in one ganglionic layer (left), but not in the other.
- B. Horizontal view, showing that climbing fibers are confined to the ganglionic layer (ggl). Thick fibers traverse along the ganglionic layer and give off branches, and thin fibers have terminal buttons.
- C. Transverse view, showing that climbing fibers traverse long distance in the ganglionic layer (ggl) in this plane. Abbreviations are the same as in Fig. 2. Scale bars = 20 μ m in A; = 30 μ m in B; = 50 μ m in C. See magenta-green version of this figure in supplementary files.

Fig. 16.

Summary diagram, showing the cell types and their connections identified in this study. Opened circles represent excitatory terminals, filled circles represent inhibitory terminals, and dashed lines represent presumed connections. Noted that axon of the granule cell projects to the molecular layer without a T bifurcation. Abbreviations: B, basal cell; cf, climbing fiber; dst, deep stellate cell; G, Golgi cell; ggl, ganglionic layer; gr, granule cell; gran, granular layer; L, large central cell; mol, molecular layer; mf, mossy fiber; P, Purkinje cell; pf, parallel fiber.