Voltage-Gated Sodium Channels in Cerebellar Purkinje Cells

of Mormyrid Fish

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Abstract

Cerebellar Purkinje cells of mormyrid fish differ in some morphological as well as physiological parameters from their counterparts in mammals. Morphologically, Purkinje cells of mormyrids have larger dendrites that are characterized by a lower degree of branching in the molecular layer. Physiologically, there are differences in electrophysiological response patterns that are related to sodium channel activity: first, sodium spikes in mormyrid Purkinje cells have low amplitudes, typically not exceeding 30mV. Second, the response to climbing fiber stimulation in mormyrid Purkinje cells does not consist of a complex spike (with an initial fast sodium spike) as in mammals, but instead it consists of an all-or-none excitatory postsynaptic potential, the so-called ‘climbing fiber response’. Because of these unique properties, we have begun to characterize mormyrid Purkinje cells electrophysiologically. In this study, we provide a description of voltage-gated Na’ channels and conductances in Purkinje cells of the mormyrid fish *Gnathonemus petersii*. Various types of Na’ channel α-subunits, namely Na,1.1, Na,1.2 and Na,1.6, have been described in rodent Purkinje cells. Using immunohistochemical techniques, we found that these subunits are present in Purkinje cells of mormyrids. To test whether these Na’ channel subunits can mediate fast inactivating and resurgent Na’ currents in *Gnathonemus* Purkinje cells, we conducted patch-clamp recordings in acutely dissociated cells and in cerebellar slices. Both types of Na’ currents could be measured in rat and fish Purkinje cells. These data show that, despite of prominent differences in electrophysiological response characteristics, Purkinje cells of rats and mormyrids share the same voltage-gated Na’ conductances.
Introduction

The cerebellum of mormyrid fish has for a long time attracted the attention of neuroanatomists due to its relative size (‘gigantocerebellum’) and its unique organization (Nieuwenhuys and Nicholson, 1967; 1969; Meek, 1992). At the cellular level, a hallmark of the mormyrid cerebellum is the unusual architecture of the Purkinje cell dendritic tree, which differs substantially from that of mammalian Purkinje cells. Their dendrites show a palisade pattern displaying very different branching characteristics. The smooth proximal dendrites do not protrude into the molecular layer and run parallel to the boundary between the ganglion cell layer (Purkinje cell layer in mammals) and the molecular layer. The proximal dendrites give rise to perpendicularly oriented dendrites, which mostly do not branch any further and run parallel to each other towards the cerebellar surface. The parallel fibers (PFs) selectively contact these distal dendrites, whereas the climbing fiber (CF) input contacts the smooth proximal dendrite. This configuration leads to a far stronger spatial separation between PF and CF synapses than that known from mammalian Purkinje neurons. The axons of mormyrid Purkinje cells are very short. They project in the ganglion cell layer itself onto efferent cells (also called eurydendroid cells) and neighboring Purkinje cells. The efferent cells represent a functional equivalent to the cerebellar nuclei neurons in mammals and provide the sole output of the mormyrid cerebellum (Nieuwenhuys and Nicholson, 1967; 1969; Meek and Nieuwenhuys, 1991; Meek, 1992). The unusual dendritic tree architecture and synaptic input organization of mormyrid Purkinje cells has motivated us to attempt to characterize these cells electrophysiologically. While the medium ganglion cells (Purkinje-like interneurons) in the mormyrid electro sensory lobe (a cerebellum-like structure) have been characterized with regard to several electrophysiological parameters and with regard to synaptic plasticity (e.g. Bell et al., 1997a+b; Han et al., 2000), the physiological description of cerebellar Purkinje
cells of mormyrid fish has only recently begun (Han and Bell, 2003). Our initial recordings focused on synaptic responses of mormyrid Purkinje cells to CF stimulation. In mammalian Purkinje cells, CF activation results in a large all-or-none response, the so-called complex spike, which consists of an initial, fast spike component followed by a series of smaller spikelets riding on top of a plateau (for review see Schmolesky et al., 2002). The fast, initial spike is generated by a somatic Na⁺ current. The following slower components have been described as resulting from the activation of dendritic Ca²⁺ conductances (Llinas and Sugimori, 1980 a+b). More recently, it has been shown that complex spike-like events can be evoked in the soma by intrasomatic depolarization or anode break stimulation (Callaway and Ross, 1997) and can even be elicited in dissociated Purkinje cells (Swensen and Bean, 2003). Thus, it is likely that the complex spike is triggered by dendritic events, but that, at least at the somatic level, the slow complex spike components are mediated by somatic Na⁺ and Ca²⁺ currents (for discussion see Schmolesky et al., 2002), which can be activated locally.

In contrast to mammals, stimulation of the CF in the cerebellum of the mormyrid fish Gnathonemus petersii does not elicit a complex spike, but rather an all-or-none ‘CF response’ that lacks obvious spike components (see below). These CF responses can occur in isolation, but they are often followed by series of ‘small spikes’, which were shown to be Na⁺ spikes, probably originating from the axon, reaching amplitudes of ≤30 mV (Han and Bell, 2003). The absence of otherwise Purkinje cell-typical complex spikes and the presence of small-amplitude Na⁺ spikes, which have not been described in other types of neurons, are the most obvious electrophysiological features that distinguish mormyrid Purkinje cells from their mammalian counterparts. Both, the unique morphological and electrophysiological properties motivated us to attempt to characterize mormyrid Purkinje cells in more detail.

Voltage gated Na⁺ channels consist of an α-subunit and one or more β-subunits. The α-subunit forms the channel pore and possesses all the main properties of the channel such as
voltage dependent gating and Na$^+$ selectivity. The β-subunits are capable of changing the channel kinetics as well as the voltage dependence of inactivation (Isom et al., 1995a+b). In the adult mammalian cerebellum, three types of Na$^+$ channel α-subunits are expressed, namely αNa,1.1, αNa,1.2 and αNa,1.6 (equivalent to rat brain I, rat brain II and Scn8a, Westenbroek et al., 1989; Vega-Saenz de Miera et al., 1997; Gong et al., 1999). Na$^+$ channel α-subunits of teleost fish, the taxonomical group to which mormyrid fish belong, show high homology to Na$^+$ channel α-subunits of rats. Goldfish αNa,1.2 and αNa,1.6 subunits, for instance, show 77% and 87% homology with αNa,1.2 and αNa,1.6 subunits in rats (Zenisek et al., 2001). In addition, two β-subunits (β1, β2) are expressed. The different subunits are heterogeneously distributed in different cell types (for review see Schaller and Caldwell, 2003). Mammalian Purkinje cells express Na$^+$ channel α-subunits Na,1.1 and Na,1.6 in both soma and dendrites (Gong et al., 1999; Schaller and Caldwell, 2003). Reports on the expression of Na,1.2 in Purkinje cells are conflicting (Black et al., 1994; Felts et al., 1997; but see Brysch et al., 1991; Gong et al., 1999).

Electrophysiologically, three tetrodotoxin (TTX) sensitive Na$^+$ conductances were described in mammalian cerebellar Purkinje cells. Recordings from Purkinje cells in slices or organotypic cultures have revealed a fast inactivating and a persistent Na$^+$ conductance (Llinas and Sugimori, 1980a; Gähwiler and Llano, 1989; Kay et al., 1998). Additionally, Raman and Bean described a third Na$^+$ current mediated by Na,1.6 channels, the ‘resurgent Na$^+$ current’ (Raman and Bean, 1997, 2001; Raman et al., 1997), which can be elicited upon repolarization after a depolarization to positive potentials. There are uncertainties about the assignment of the different subunit types to particular currents. Recordings from Purkinje cells of Na,1.6 knock-out mice indicate that Na,1.6 channels mediate large parts of the resurgent Na$^+$ current (Raman et al., 1997). The fast, inactivating Na$^+$ currents and the persistent Na$^+$ currents were reduced in those mutant mice as well, but to a lower degree.
These observations indicate that Na\textsubscript{1.6} channels participate in all three types of currents, but that Na\textsubscript{1.1} and Na\textsubscript{1.2} channels are involved in the fast, inactivating Na\textsuperscript{+} currents and/or the persistent Na\textsuperscript{+} currents as well ((for discussion see Kay et al., 1998; Schaller and Caldwell, 2003).

Na\textsubscript{1.6} mediated resurgent Na\textsuperscript{+} currents are pleomorphic in nature. While in Na\textsubscript{1.6} null mutant mice resurgent currents are normally absent or very small in Purkinje cells (Grieco and Raman, 2004), subthalamic nucleus neurons from Na\textsubscript{1.6} null mutants show considerable resurgent Na\textsuperscript{+} currents (37% of wildtype) (Do and Bean, 2004). Another example illustrating the pleomorphic nature is provided by CA3 pyramidal neurons and motor neurons, which do express Na\textsubscript{1.6} Na\textsuperscript{+} channels but lack resurgent Na\textsuperscript{+} currents (Raman and Bean, 1997; Garcia et al., 1998; Pan and Beam, 1999). Resurgent Na\textsuperscript{+} currents mediated by Na\textsubscript{1.6} channels can recover from inactivation at relatively depolarized potentials (Raman and Bean, 1997; Khaliq et al., 2003). This feature enables them to accelerate spike firing during bursts. It is therefore likely that resurgent Na\textsuperscript{+} currents also contribute to the late complex spike components, which can reach frequencies exceeding 200Hz. Although there are still gaps in our understanding of which conductances are mediated by which types of Na\textsuperscript{+} channel \(\alpha\)-subunits, it can be shown that alterations in the expression pattern of Na\textsuperscript{+} channel \(\alpha\)-subunits lead to changes in the firing pattern of Purkinje cells. For example, Purkinje cells from mice lacking Na\textsubscript{1.6} channels do not only show a reduction in resurgent currents, but also diminished repetitive spike firing (Raman et al., 1997). Biologic introduction of Na\textsubscript{1.8} cDNA into Purkinje cells reduced the number of spikes in conglomerate action potentials evoked by depolarizing current pulses (Renganathan et al., 2003), thus altering a characteristic feature of Purkinje cell electrophysiology. These observations emphasize that the expression profile of voltage-dependent Na\textsuperscript{+} channels provides an important component of a complete electrophysiological
characterization of neurons, even if the expression of a certain type of subunit does not allow to reliably predict associated conductances.

To characterize the functional expression of Na\(^+\) channel \(\alpha\) subunits, we applied immunohistochemistry to describe their distribution and performed voltage-clamp recordings to test for the presence of various types of voltage-gated Na\(^+\) conductances. All immunohistochemical as well as electrophysiological experiments were not only performed in Purkinje cells of the mormyrid fish *Gnathonemus petersii*, but also in rats to obtain reference values for comparison and to investigate the debated presence of Na,1.2 Na\(^+\) channels in rat Purkinje cell membranes.
Materials and methods

Animals
Sprague-Dawley rats (Harlan, Netherlands), aged P18-P28 were housed in filtertop cages. Mormyrid fish of the species *Gnathonemus petersii* (obtained from a local fish dealer) were wild-caught and kept in standard aquaria. All experiments described were approved by the Erasmus Medical Center animal care and use committee.

Immunohistochemistry
Mormyrid fish and rats were anaesthetized with 0.20 mmol/l Eugenol and 0.2 ml Nembutal, respectively, and perfused with 4% paraformaldehyde in 0.02 M phosphate buffer (PB). Brains were removed, postfixed and rinsed overnight at 4 °C in 0.1 M PB, containing 10% sucrose. Embedding of the brains in gelatin was done as described by Groenewegen and Voogd (1977). 40 µm thick sections were cut and collected in 0.1 M PB. Sections were rinsed in Tris buffered saline (TBS) and pre-incubated for 1 hour at 4 °C in 10% normal horse serum (NHS) and 0.5 % triton in TBS.

Avidine biotine complex (ABC) staining
Sections were incubated with primary antibody for 48 to 72 hours at 4 °C in incubation buffer 1 (IB1) containing: 2% NHS and 0.4% triton in TBS and rinsed (TBS). Secondary antibody was added for 1.5 to 2 hours at room temperature in IB1. Sections were rinsed again, after which the biotin labeled secondary antibody was conjugated with Avidine from a Vectastain ABC kit (Vector, Burlingame, CA, USA). Sections were rinsed (TBS, Tris) and stained for 15 minutes using diaminobenzidine (DAB). Finally sections were rinsed again (Tris, PB), put on slides, dried and coversliped.
Fluorescent labeling

Sections were incubated 48 hours with primary antibody at 4°C in incubation buffer 2 (IB2) containing: 1% NHS and 0.4% triton in TBS and rinsed (TBS). Sections were then incubated for 90 minutes with secondary antibody in IB2 and rinsed (TBS). Finally, they were mounted on slides and coverslipped.

Primary antibodies used were: Rabbit anti-brain type I Na⁺ channel (Na,1.1) directed against peptide (KY)TAS EHSRE PSAAG RLSD, corresponding to residues 465-481 of rat Na,1.1 intracellular loop between I and II domains (accession P04774), rabbit anti-Na,1.2 directed against peptide (KY)ASA ESRDF SGAGG IGVFS E, corresponding to residues 467-485 of rat Na,1.2 intracellular loop between I and II domains (accession P04775), rabbit anti-Scn8α (Na,1.6) directed against peptide CIANH TGVDI HRNGD FQKNG, corresponding to residues 1042-1061 of rat Scn8α intracellular loop between II and III domains (accession AAC26014) (Alomone labs, Jerusalem, Israel) and rabbit anti-IP₃ receptor subtype I (Calbiochem, Amsterdam, Netherlands). Secondary antibodies used were: Biotinylated goat anti Rabbit (Bio-Gar) (Vector, Burlingame, CA, USA) and fluorescein-isothiocyanate (FITC) labeled donkey anti rabbit (FITC-Dar) (Jackson, Amsterdam, Netherlands). All other drugs were purchased from Sigma.

Control experiments for antibody staining

The specificity of the immunohistochemical procedure was examined by performing parallel incubations in which the primary antibody was omitted, or pre-adsorbed. Before pre-adsorption testing we first determined the antibody concentration at which staining was just visible. For all three Na⁺ channel primary antibodies used this concentration was 1:100. We then doubled the primary antibody concentration and 1h before incubating the sections, as
suggested by Saper and Sawchenko (Saper and Sawchenko, 2003), we added 50 µM/ml of antigen against which the primary antibody was raised. We used three different control antigens (rat) for the three Na⁺ channel primary antibodies (Alomone labs, Jerusalem, Israel).

Western blots
Rats and mormyrid fish were anaesthetized with halothane or 0.20 mmol/l eugenol, respectively, and decapitated, brains were removed and put on ice in 320 mM sucrose with protease inhibitor cocktail (Boehringer-Mannheim). Brains were homogenized and centrifuged at 2000 g for 5 minutes. Supernatant was collected and centrifuged at 100,000 g for 1 h at 4°C after which the pellet was resuspended in 320 mM sucrose with protease inhibitor cocktail to a final concentration of 2000 µg per 10 µl and stored at -80°C. Samples were mixed with sample-buffer and samples plus markers (Biorad) were heated for 10 minutes at 70°C. 10 µl of sample per lane was applied to a 7% polyacrylamide gel. Gels were run for 1.5 h at 30 mA/gel under standard conditions. After running the gel was completed, proteins were transferred onto a nitrocellulose membrane for 1.5 h using standard wet-blot techniques at 100mA per gel. Blots were then blocked with 5% non-fat dry milk in PBS and 0.025% Na azide for 2 h at room temperature. Blots were incubated with primary antibody (anti-Na,1.1, 1.2 and 1.6, final concentration 1:100, Alomone labs, Jerusalem, Israel) for 2 h at room temperature in blocking solution. Blots were then washed 4 times for 10 minutes with PBS containing 0.1% tween 20. Incubation with secondary antibody (SwaR-HRP (Swine anti Rabbit conjugated with Horse Radish Peroxidase) 1:4000, Dako, Denmark) was done at room temperature for 1 h. After the blot was washed again ECL was performed using a commercial kit (Amersham Bioscience). Film (Kodak) was developed on a Kodak film processor.
Photography

Photographs (Fig. 2A and 2B, Fig. 3,4 and 5 panels A-F) were taken using a digital camera (Leica) mounted on a brightfield microscope (Leica). Brightness and contrast were enhanced to comparable levels using the camera’s software package (Leica) and microscope settings before the pictures were taken. Fluorescent images (Fig. 2C,D) were taken using a confocal microscope (Zeiss). The image in Fig 2D was constructed from a 15 image Z-stack using LSM photo software (Zeiss). Films developed for our Western blot experiments (Fig 3G,4G and 5G) were scanned using a HP flatbed scanner.

Electrophysiology

The mormyrid cerebellum is subdivided into a valvula, a central corpus and a caudal lobe (see Meek, 1992). The corpus cerebelli consists of four central lobes, C1 to C4. Our recordings from mormyrid Purkinje cells were restricted to these central lobes. In the rat, all experiments were performed using the cerebellar vermis. All recordings were done at room temperature.

Slice recordings (Fig.7): Rats and mormyrid fish were anaesthetized with halothane or 0.20 mmol/l eugenol, respectively, and decapitated, brains were removed, cut into 200 µm thick sagittal slices and immersed in standard artificial cerebrospinal fluid (ACSF) containing in mM: 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄ and 10 D-glucose, bubbled with 95% O₂ and 5% CO₂. Following a recovery period of at least 1 hour, slices were placed in a submerged chamber and perfused at a flow rate of 1.6 ml/min with either bubbled ACSF (Fig. 1) or rACSF (“resurgent” artificial cerebrospinal fluid) containing in mM: 122 NaCl, 5 TEA-Cl, 2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄, 10 D-glucose and 0.30 CdCl₂ to block most Ca²⁺ and K⁺ currents (Fig. 6,7). Acutely dissociated cells (Fig.6): Slices were prepared as mentioned. Rat cerebellar slices were then treated with 3 mg/ml
protease XXIII in dissociation buffer (DB) containing in mM 82 Na$_2$SO$_4$, 30 K$_2$SO$_4$, 5 MgCl$_2$, 10 HEPES and 10 glucose (buffered to pH 7.4 using NaOH) for 7 minutes at 37 degrees with oxygen blown over the surface. Tissue was washed in warm oxygenated DB containing 1mg/ml BSA + 1mg/ml trypsin inhibitor and was allowed to cool to room temperature. The tissue was then triturated in DB and maintained at room temperature with oxygen blown over the surface in Tyrode’s solution containing in mM 150 NaCl, 4 KCl, 2 CaCl$_2$, 2MgCl$_2$, 10 HEPES and 10 glucose (buffered to pH 7.4 using NaOH). Cells were used within four hours after dissociation (Raman and Bean, 1997). Fish slices were incubated for 15 minutes at room temperature using 40 U/ml Papain in eagle’s MEM incubation buffer (IB) containing in mM 10 HEPES, 1 Cysteine, 0.5 EDTA and 5 Na-acetate (pH 7.2). Slices were then washed and triturated in IB, and kept in recording solution in the recording chamber to settle (Afshari et al., 2004). All recordings from acutely dissociated cells were made in rACSF. Recordings were performed using the visualized whole-cell patch clamp technique with a Zeiss Axioskop FS and an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany). Recording electrodes (resistance 3-5 MΩ) were filled with a solution containing in mM: 9KCl, 10KOH, 120K$^+$ gluconate, 3.48 MgCl$_2$, 10 HEPES, 4 NaCl, 4 Na$_2$ATP, 0.4 Na$_3$GTP and 17.5 sucrose (Fig. 1A-C) or, in mM: 128 CsOH, 111 gluconic acid, 4 NaOH, 10 CsCl, 2 MgCl$_2$, 10 HEPES, 4 Na$_2$ATP, 0.4 Na$_3$GTP and 30 sucrose (Fig. 1D-F, Fig. 6+7; Hansel and Linden, 2000). Both types of internal saline were pH-adjusted (pH 7.25). Currents were filtered at 3kHz and digitized at 8kHz using Pulse software. For extracellular stimulation (Fig. 1), standard patch pipettes were filled with external saline. Climbing fibers were stimulated in the granule cell layer. Voltage-step protocols used were either: a 20 millisecond depolarizing step from -90 to +30 mV followed by repolarizing steps to potentials between 0 and -60 mV or a depolarizing ramp, 0.1 mV/millisecond from -90 to +30 mV followed by repolarizing steps between +20 and -60 mV (Raman and Bean, 1997). Traces recorded in rACSF containing 300 nM TTX
were subtracted from traces recorded in rACSF alone to isolate TTX-sensitive Na⁺ currents from e.g. capacitive and leak currents (Fig. 6,7). Directly after forming a giga-seal the fast capacitance was corrected using the automatic capacitance compensation function embedded in the amplifier window of the HEKA Pulse software package. After brake in (whole cell configuration) the slow capacitance was compensated the same way. All drugs were purchased from Sigma.
Results

Synaptic responses to CF stimulation

In mammalian Purkinje cells, CF activation results in the firing of a complex spike. Figure 1A shows a complex spike recorded from a rat Purkinje cell. The complex spike is characterized by an initial somatic Na\(^+\) spike, followed by a slow plateau potential with small spikelets on top. The plateau potential could result from Ca\(^{2+}\) currents and/or non-inactivating Na\(^+\) currents in the Purkinje cell soma and proximal dendrites (Llinas and Sugimori, 1980a+b; for review see Schmolesky et al., 2002). The origin of the small spikelets is still not resolved. While the complex spike is initiated by the activation of dendritic \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and subsequent local Ca\(^{2+}\) spike activity, it is likely that the spikelets, as recorded in the soma, are mediated by somatic Ca\(^{2+}\) and Na\(^+\) currents. Resurgent Na\(^+\) currents might facilitate the high frequency discharge that characterizes the late complex spike components. The climbing fiber response of mormyrid fish (Gnathonemus petersii) differs substantially from its mammalian counterpart in several aspects (Fig. 1B+C). The initial component is an all-or-none excitatory postsynaptic potential (EPSP; the all-or-none character is shown in Fig. 1B), which is sometimes followed by one or more small spikes which are thought to be axonal Na\(^+\) spikes that do not invade the soma (Han and Bell, 2003). These small spikes may or may not occur (for examples of CF responses without and with small spikes see Figs. 1B+C, respectively) and thus cannot be considered as a CF response component. CF evoked excitatory postsynaptic currents (EPSCs) reverse typically around +12 mV in both rat and mormyrid fish Purkinje cells (Fig. 1D+E). The amplitudes of mormyrid fish EPSCs are generally smaller than those recorded in rat Purkinje cells (Fig. 1D+E). CF EPSCs of both species show similar rise time kinetics when traces above and below the reversal potential were scaled to the same amplitude after the positive currents were reversed (Fig. 1F). Since voltage-gated Na\(^+\) channels are critically
involved in the generation of complex spikes, and CF responses differ substantially between rat and mormyrid fish, we wanted to determine the expression patterns of the three mammalian Na\textsuperscript{+} channel $\alpha$ subunits in mormyrid fish to better understand the molecular and cellular basis of the unique electrophysiological features of their Purkinje cells.

**Immunohistochemistry**

In mormyrid fish, cerebellar Purkinje cells can be confused with efferent cells or stellate cells, which have a roughly similar palisade pattern of their dendritic trees in the molecular layer. Reliable criteria for the identification of Purkinje cells are a particularly low degree of dendritic branching and the resulting regularity in appearance, the relative thickness of dendrites, the presence of dendritic spines and the superficial position of the somata within the ganglion cell layer (Meek and Nieuwenhuys, 1991). In addition, IP\textsubscript{3}-receptor subtype I is known to be selectively expressed in Purkinje cells (Sharp et al., 1999; Koulen et al., 2000). To obtain reference images for the identification of Purkinje cells, we used an antibody against the IP\textsubscript{3}-receptor subtype I. The DAB staining pattern for IP\textsubscript{3}-receptor subtype I was similar in both rat and mormyrid fish (Fig. 2A,B). In rat sections, the Purkinje cells are easily identified by the unique shape of their dendritic trees. It is obvious from figure 2A that the staining was selective for Purkinje cells. In sections of mormyrid fish, the identity of the stained cells was confirmed by the presence of spines (Fig. 2D), which we could visualize using fluorescent secondary antibodies (Fig. 2C,D). The presence of spines allows for an unambiguous identification of these cells as Purkinje cells, because the other two types of neurons with palisade-shaped dendrites are aspiny (Meek and Nieuwenhuys, 1991; Meek, 1992). Thus, in both rat and fish sections the staining for IP\textsubscript{3}-receptor subtype I was Purkinje cell-specific.
To characterize the distribution pattern of Na$_{\text{v}}$1.1 channels, we immunostained cerebellar sections obtained from rats (Fig. 3A,B) and from mormyrid fish (Fig. 3C,D). In rat sections, Purkinje cell somata are stained and there is some staining in the molecular layer that, however, is too weak to identify dendritic structures. In the fish sections, there is a staining of small and larger somata in the ganglion cell layer as well and also a similarly weak staining in the molecular layer. Figures 3C+D show pictures taken from the central lobe C2. In contrast to lobes 3 and 4, lobes 1 and 2 are known to have a layer of stellate cells external to the layer of Purkinje cells (Han and Bell, personal communication). In Figure 3D, smaller, more superficial somata can be distinguished from larger somata. Thus, it is likely that somata of both stellate and Purkinje cells were stained. In contrast to the staining for Na$_{\text{v}}$1.1 channels, antibodies against Na$_{\text{v}}$1.2 channels let to a strong staining in rat Purkinje cells in both somata and dendrites (Fig. 4A,B). Similarly, somata and dendrites of *Gnathonemus* Purkinje cells were heavily stained (Fig. 4C,D). The dendrites could be clearly distinguished and the identification of Purkinje cells was based on the criteria as mentioned above. A very similar staining pattern emerges from the antibody staining against Na$_{\text{v}}$1.6 channels. In rat sections, there is a strong staining in somata and dendrites of Purkinje cells (Fig. 5A,B). A similar distribution can be seen in fish sections, where Na$_{\text{v}}$1.6 channels are also expressed in Purkinje cell somata and dendrites (Fig. 5C,D). These results show that Purkinje cells in rats and mormyrid fish show the same expression pattern of Na$^+$ channel $\alpha$ subunits. Na$_{\text{v}}$1.1 channels also appeared to be expressed in somata in the granule cell layer (these could be somata of Golgi cells, unipolar brush cells or a subset of granule cells), whereas Na$_{\text{v}}$1.2 and 1.6 were found to be not, or very lightly, expressed in the granule cell layer (Fig. 3,4,5). Figures 3,4 and 5 show, in panel E and F, that when the primary antibody directed against the respective Na$^+$ channel was adsorbed to the antigen before incubation (panel E) or omitted (panel F), no, or very weak, staining could be detected in mormyrid slices compared to panels C and D of
figures 3, 4 and 5. When we compare panel E and F we can conclude that the very weak staining that is still found after adsorption and omission of the antibody can be fully attributed to weak unspecific staining of the secondary antibody used. In addition, Western blot analysis (Fig. 3, 4, 5 G) shows that antibodies directed against Na\textsubscript{v}1.1, Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6 stain proteins of the expected molecular size in both fish and rat brain membrane preparations. These results show that the Na\textsuperscript{+} channel antibodies used in this study are specific for the antigen they are directed against (Fig. 3, 4, 5 E) and that these antibodies react to proteins of the appropriate size in mormyrid fish brain membrane preparations (Fig. 3, 4, 5 G). The secondary antibody used does only very weakly stain the cerebellar sections to a degree that does not interfere with the interpretation of our results (Fig. 3, 4, 5 F). Purkinje cells of both species express the same subset of Na\textsuperscript{+} channels in comparable densities at comparable sites. To examine whether the immunohistochemically characterized Na\textsuperscript{+} channels are functional and to compare Na\textsuperscript{+} conductances in Purkinje cells of rats and mormyrid fish, we set out to study different types of Na\textsuperscript{+} conductances using patch-clamp electrophysiological recordings.

*Electrophysiological characterization of Na\textsuperscript{+} conductances*

We first recorded from dissociated neurons to minimize space-clamp limitations and thus to allow for a characterization of current properties. Voltage steps (20 millisecond depolarizing step from -90 to +30 mV; Fig. 6A) resulted in a fast inactivating TTX sensitive Na\textsuperscript{+} current in dissociated rat Purkinje cells (n=6, Fig. 6B,F). The displayed traces show the TTX sensitive current components after subtraction of the currents that remained when TTX was bath-applied. In the following, the TTX sensitive currents remaining after subtraction are labelled as ‘TTX subtracted’. In dissociated mormyrid Purkinje cells, we found a similar fast inactivating current (n=6, all TTX subtracted; Fig. 6C,F). Sodium currents were corrected for
input capacitance (16.6 ± 1.1 pF (±SEM) for rat (n=5) and 9.8 ± 0.7 pF (±SEM) for mormyrid (n=5) Purkinje cells) and plotted as current density on a pA/pF scale. This was done to correct for the smaller size of mormyrid Purkinje cell somata. The fast inactivating Na\(^+\) current reached 189 ± 57 pA/pF (±SEM) with a decay time constant of 0.31 ± 0.049 ms (±SEM) in rat Purkinje cells (n=6, all TTX subtracted) and 179 ± 59 pA/pF (±SEM) with a decay time constant of 0.47 ± 0.056 (±SEM) ms in mormyrid Purkinje cells (n=6, all TTX subtracted, p>0.05, Mann-Whitney U test) (Fig. 6H+L). Fast inactivating current rise times also did not differ between species (0.38 ± 0.052 ms (±SEM) for rat and 0.39 ± 0.008 ms (±SEM) for mormyrid dissociated cells, p>0.05, Mann-Whitney U test) (Fig 6J). Our data from rat dissociated Purkinje cells are very similar to those previously obtained from isolated rat Purkinje cell somata (Raman and Bean, 1997). To test for the presence of resurgent Na\(^+\) currents in the dissociated cell preparations, the depolarizing voltage steps were followed by repolarizing steps to potentials between 0 and -60 mV (rat n=6 all TTX subtracted; fish n= 6; all TTX subtracted) (Fig 6A). Resurgent Na\(^+\) currents were elicited in mormyrid fish and rat Purkinje cells during repolarization steps (Fig. 6 B,C,E), which were very similar to resurgent Na\(^+\) currents previously described in rat and mouse cerebellar Purkinje cells using the same protocol (Raman and Bean, 1997; Raman et al., 1997). In rat Purkinje cells, the amplitude of the resurgent current was, at peak, 30.6 ± 8.7 pA/pF (±SEM) with a rise time of 4.22 ± 0.35 ms (±SEM) and a decay time constant of 17.1 ± 2.01 ms (±SEM) (n=6, all TTX subtracted, step protocol, -30 mV) (Fig 6 G, I, K). In mormyrid Purkinje cells, the resurgent Na\(^+\) current reached 25.6 ± 4.0 pA/pF (±SEM) with a rise time of 4.77 ± 0.54 ms (±SEM) and a decay time constant of 10.24 ± 1.29 ms (±SEM) (n=6, all TTX subtracted, step protocol, -40 mV) (Fig 6 G, I, K). Resurgent current peak size, rise and decay times were all not significantly different between rat and mormyrid Purkinje cells at all voltage steps (p>0.05, Mann-Whitney U test). There was also no difference in the input resistance (rats: 104.9 ± 13.1 MΩ; n=5; fish:
97.1 ± 13.3 MΩ; n=4; p>0.05; Mann-Whitney U test; Fig. 6D). Our observations suggest that the types of conductances described here are of somatic origin. This assumption is supported by the immunohistochemical data. A caveat is, however, that the dissociated Purkinje cells often still contain axon and dendrite stumps. Therefore, it is not possible to exclude contributions from these structures to the recorded conductances.

While recordings from dissociated neurons provide the best technical approach to characterize Na⁺ currents, they are limited by difficulties to distinguish different types of neurons. This aspect is particularly relevant for the mormyrid cerebellum, because here Purkinje cells and efferent cells are quite similar and are, in fact, indistinguishable after dissociation. Therefore, we performed an additional series of recordings from Purkinje cells in slices to qualitatively confirm the observations described above. Purkinje cells were identified by the superficial position of their somata in the ganglion cell layer (Meek and Nieuwenhuys, 1991). Voltage steps (20 millisecond depolarizing step from -90 to +30 mV; Fig. 7A) resulted in a fast inactivating TTX sensitive Na⁺ current in rat Purkinje cells (n=15, of which 3 were TTX subtracted; Fig. 7B). Sodium currents were corrected for input capacitance (1013 ± 195 pF (±SEM) for rat (n=5) and 833 ± 92 pF (±SEM) for mormyrid (n=5) Purkinje cells) and plotted on a pA/nF scale. The fast current component was followed by a slower, low amplitude current. In mormyrid Purkinje cells, we found a similar biphasic current (n=10, of which 5 were TTX subtracted; Fig. 7D). The fast inactivating Na⁺ current differed (p < 0.05, Mann-Whitney U test), in amplitude, but not in rise or decay time constant (p > 0.05, Mann-Whitney U test, measured at 50% decay) between the two species, even after compensation for cell capacitance. This difference might be due to the poor space clamp conditions in intact Purkinje cells which makes a good estimate of cell capacitance virtually impossible. The current reached 3.78 ± 0.71 nA/nF (±SEM) with a decay time constant of 0.80 ± 0.19 ms (±SEM) in rat Purkinje cells (n=3, all TTX subtracted) and 1.31 ± 0.0.62 nA/nF (±SEM) with
a decay time constant of $1.02 \pm 0.24$ ms ($\pm$SEM) in mormyrid Purkinje cells (n=6, all TTX subtracted) (Fig 7F+G). Rise times were ($0.42 \pm 0.066$ ms ($\pm$SEM) in rats and $0.37 \pm 0.048$ ms ($\pm$SEM) in mormyrids) (Fig 7G). As these recordings were obtained from intact Purkinje cells and not from isolated somata, it is possible that inadequate space clamp in some cells allowed for voltage escape during the depolarizing step (for a discussion of the space clamp problem see Häusser, 2003). Despite of this technical limitation that makes a reliable quantification difficult to achieve, these data from intact slices are similar to those obtained in our dissociated cell experiments and to those previously obtained from isolated rat Purkinje cell somata (Raman and Bean, 1997). They furthermore strongly resemble fast current recordings recently obtained from intact Purkinje cells in slices (Afshari et al., 2004). An interesting side note is that after dissociation, the input capacitance decreases to a comparably larger degree than the Na$^+$ current amplitudes (in both rat and mormyrid Purkinje cells), providing further evidence that the Na$^+$ channel density is particularly high in the soma (see also distribution of Na$\text{v}1.1$ channels in Fig.3). To test for the presence of resurgent Na$^+$ currents, the depolarizing voltage steps were followed by repolarizing steps to potentials between 0 and $-60$ mV (rat n=15 of which 3 were TTX subtracted; fish n= 10; 5 TTX subtracted) (Fig 7A). Alternatively, we used ramp protocols (0.1 mV/millisecond from $-90$ to $+30$ mV followed by repolarizing steps between $+20$ and $-60$ mV, fish n=6; rat n=7; all TTX subtracted; Fig. 7C, E, H). Both protocols elicited resurgent Na$^+$ currents in mormyrid fish and rat Purkinje cells during repolarization (Fig. 7C+E), which were resembling the resurgent Na$^+$ currents we recorded in dissociated cells and those previously described in rat and mouse dissociated cerebellar Purkinje cells using the same protocol (Raman and Bean, 1997; Raman et al., 1997). The resemblance between our slice data and previously published work on slices (Afshari et al., 2004) is again striking. In rat Purkinje cells, the amplitude of the resurgent current was $110.32 \pm 36.19$ pA/nF($\pm$SEM) (n=4, all TTX subtracted, ramp protocol, $-30$ mV)
(Fig 7H). In mormyrid Purkinje cells, the resurgent Na\(^+\) current reached 219.30 ± 82.33 pA/nF (±SEM) (n=4, all TTX subtracted, ramp protocol, -30 mV) (Fig 7H) (p > 0.05, Mann-Whitney U test). No attempt was made to quantify the voltage-dependence, or kinetics, of the resurgent currents in slices because the voltage at which the peak amplitude was measured, and the kinetics of the resurgent current, varied from cell to cell probably due to space clamp problems. In all recordings, however, the voltage-dependence of the resurgent current was clearly seen. There was no difference in the input resistance between rat and mormyrid Purkinje cells in slices (rat: 84.8 ± 18.5 MΩ; n=5; fish: 81.3 ± 13.1 MΩ; n=5; p > 0.05, Mann-Whitney U test; data not shown).

The immunohistochemical and electrophysiological data shown above indicate that rat and mormyrid Purkinje cells qualitatively share the same set of Na\(^+\) conductances. Nevertheless, CF stimulation does not evoke complex spikes in mormyrid Purkinje cells. Isolated somata of rat Purkinje cells can still fire complex spikes. The underlying Na\(^+\) currents have been examined in the dynamic clamp configuration, which allows to apply a complex spike as the command potential and to record isolated currents in voltage-clamp mode. In this configuration, rat Purkinje cells are able to elicit Na\(^+\) currents at the high frequency that is typical for the spike components of a complex spike (Raman and Bean, 1997). It was suggested that this ability to fire conglomerate action potentials is due to resurgent Na\(^+\) currents, which recover rapidly from inactivation. To test whether the observed differences in CF responses between rat and mormyrid Purkinje cells result from differences in the ability to repetitively activate Na\(^+\) conductances at the frequency required to fire a complex spike, we applied the dynamic clamp technique to dissociated rat and mormyrid Purkinje cells (Fig. 8). The complex spike used as a command potential was recorded from an intact Purkinje cell in a rat cerebellar slice. Dissociated Purkinje cells from both rats (Fig. 8A; n=7; 4 TTX subtracted) and mormyrid fish (Fig. 8B; n=7; 3 TTX subtracted) were able to respond to the
spike components with transient Na⁺ currents. A notable exception in both rat and fish Purkinje cells was the first small spikelet of the complex spike waveform. This first spikelet did not elicit a separate current transient (Fig. 8A+B). Such failure was not observed by Raman and Bean (1997), but it should be noted that the frequency of spike components in the command potential applied here was higher, which could explain the current transient failure, particularly after the large initial transient. It is obvious from the traces shown in Fig. 8 that the initial Na⁺ current recorded from the rat Purkinje cell is larger than that recorded from the mormyrid Purkinje cell, although in both recordings the same command potential was applied. However, the absence of qualitative differences in the command potential-evoked currents between rat and mormyrid Purkinje cells shows that the somata of mormyrid Purkinje cells possess the Na⁺ conductances required to support repetitive action potential firing as well. Thus, our somatic recordings did not reveal any differences in Na⁺ conductances that could provide an explanation for an absence of synaptically evoked complex spikes in mormyrid fish.
Discussion

The dendritic tree architecture of Purkinje cells and their electrophysiological characteristics differ substantially between mormyrid fish and mammals. These differences motivated us to better characterize Purkinje cells of the mormyrid fish *Gnathonemus petersii* using immunohistochemical as well as electrophysiological approaches. In this initial study, we focussed on Na\(^{+}\) currents, because the most striking electrophysiological differences are related to Na\(^{+}\) spike activity, namely a) unusually low Na\(^{+}\) spike amplitudes and b) the absence of complex spikes, including their fast, initial Na\(^{+}\) spike component.

Our immunohistochemical experiments show that the Na\(^{+}\) channel α-subunits Na\(_{1.1}\), 1.2 and 1.6 are present in comparable densities and locations in the mormyrid and rat cerebellum (Fig. 3,4,5). Na\(_{1.1}\) is expressed in Purkinje cell somata, but the staining in the molecular layer is weak and does not allow to resolve dendrites. In mammalian Purkinje cells, Na\(^{+}\) action potentials do not backpropagate into the dendrite, which is partially due to a low dendritic Na\(^{+}\) channel density (Stuart and Häusser, 1994). Our observations raise the question whether or not Na\(_{1.1}\) channels need to be present at a certain density to allow action potentials to backpropagate into the dendrites. Of the three types of Na\(^{+}\) channel α-subunits, only Na\(_{1.1}\) is also expressed in somata in the granule cell layer of both species. In contrast to the Na\(_{1.1}\) subunits, Na\(_{1.2}\) and 1.6 were found to be densely expressed in somata of both rat and mormyrid Purkinje cells as well as in the molecular layer. We cannot rule out the possibility that the staining in the molecular layer reflects the expression of these Na\(^{+}\) channel α-subunits in PF terminals. Staining of these subunits has been described in granule cell ascending axons and parallel fibers (Schaller and Caldwell, 2003). In our immunohistochemical experiments, we did, however, not see staining of granule cell somata or axons above background levels.

The expression of Na\(_{1.2}\) in mammalian Purkinje cells has been debated (Black et al., 1994; Felts et al., 1997; but see Brysch et al., 1991; Gong et al., 1999; for review see Schaller and
Caldwell, 2003). Our data indicate that Na\(_{1.2}\) is expressed in somata and dendrites of rat Purkinje cells and thus support the findings of Waxman and colleagues (Black et al., 1994; Felts et al., 1997). Na\(_{1.6}\) channels were strongly expressed in somata and dendrites of rat and fish Purkinje cells as well. So far, the resurgent Na\(^+\) current, which is attributed to Na\(_{1.6}\) channels, has been described on the basis of somatic patch-clamp recordings (Raman and Bean, 1997, 2001). Our data suggest that this current exists in the dendrites as well. Next, we conducted voltage-clamp experiments to investigate whether the Na\(^+\) channels found to be expressed in the mormyrid cerebellum could mediate the same set of voltage-gated conductances as the Na\(^+\) channels expressed in rat Purkinje cells. The present results show that a depolarizing voltage step activates a TTX-sensitive, fast inactivating Na\(^+\) current in dissociated Purkinje cells of both rats and mormyrid fish, which is followed by a low amplitude tail component in slice recordings. While the fast, inactivating current was clearly present in both preparations, it reached higher amplitudes in rat than in mormyrid Purkinje cells in slices. Using a voltage step (dissociated cells and slices) or a ramp (slices only) protocol, we could demonstrate a TTX-sensitive resurgent Na\(^+\) current in both rats and fish, which strongly resembled the resurgent Na\(^+\) current described earlier (Raman and Bean, 1997; Afshari et al., 2004). The resurgent currents had indistinguishable amplitudes in rat dissociated cells compared to mormyrid cells after capacitance compensation (p > 0.05, Mann-Whitney U rank test) and these amplitudes were well in the range of data published earlier (Raman and Bean, 1997; Afshari et al., 2004) Rise and decay time constant ranges in the two species were overlapping and were also similar to what has been published before (Raman and Bean, 1997; Afshari et al., 2004). The recordings in slices were added to demonstrate that the resurgent currents are indeed present in Purkinje cells (as they cannot be distinguished with certainty from similar cells once being dissociated) under more physiological conditions (without their dendrites being cut). Resurgent Na\(^+\) currents have so
far been described in only a limited number of different types of neurons. In the cerebellum, they were described in Purkinje cells (Raman and Bean, 1997), unipolar brush cells (Mossadeghi and Slater, 1998; Afshari et al., 2004), deep cerebellar nuclei cells (Afshari et al., 2004) and granule cells (D’Angelo et al., 2001; Afshari et al., 2004). Our results show that mormyrid Purkinje cells, which otherwise differ in several morphological and physiological parameters from their mammalian counterparts, can be added to the list.

We have performed all immunohistochemical and electrophysiological experiments in slices or dissociated neurons from both rats and mormyrid fish to allow for a direct comparison. While there are obvious differences in electrophysiological response characteristics, we did not detect differences in the expression pattern of Na\textsubscript{1.1}, Na\textsubscript{1.2} or Na\textsubscript{1.6} subunits, or in fast, inactivating or resurgent Na\textsuperscript{+} currents. Moreover, the dynamic clamp recordings shown in Fig. 8 indicate that dissociated rat and mormyrid Purkinje cells (which basically consist of isolated somata) respond to a complex spike applied as the command potential with repetitive Na\textsuperscript{+} current transients. Thus, rat and mormyrid Purkinje cells also show no differences in their ability to activate Na\textsuperscript{+} conductances at high enough frequencies to support complex spike firing. While these observations allow us to conclude that the different electrophysiology is not due to differences in the functional expression pattern of these three types of Na channel \(\alpha\) subunits, they do not allow any conclusion what other parameters are causing these differences instead. Possible candidates are differences in the expression pattern of voltage-gated K\textsuperscript{+} or Ca\textsuperscript{2+} channels, or the different dendrite morphologies (see also Mainen and Seijnowski, 1996; Vetter et al., 2001).

Two types of electrophysiological differences were of particular interest to us, namely the absence of complex spikes in mormyrid Purkinje cells and their low-amplitude Na\textsuperscript{+} spikes. The complex spike received attention following recent demonstrations of synaptic plasticity at the CF synapses, involving long-term changes of slow complex spike components (Hansel
and Linden, 2000; Hansel et al., 2001; Weber et al., 2003). These results stimulated some interest in the ionic composition of the slow complex spike components (for a discussion see Schmolesky et al., 2002). It has been suggested that resurgent Na$^{+}$ currents might be characteristic for types of rapidly firing neurons (Afshari et al., 2004) and might allow Purkinje cells to generate the high-frequency spikelets that make up the late complex spike components (Raman et al., 1997; Schmolesky et al., 2002). Our results show that, while mormyrid Purkinje cells do not fire complex spikes, they still show resurgent Na$^{+}$ currents. What other parameters might then cause the absence of complex spikes in mormyrid Purkinje cells? As we argued above, the unique dendrite morphology might be involved, but at this point we simply do not understand the impact of this parameter on spike patterns sufficiently well to argue what specific features of mormyrid Purkinje cell dendrites would prevent complex spike firing. Moreover, burst firing (as in complex spikes) can also be evoked in dissociated Purkinje cells (Swensen and Bean, 2003). This observation does not exclude an impact of the dendrite morphology on the occurrence or waveform of complex spikes, but it shows that this characteristic all-or-none response of mammalian Purkinje cells can be evoked in isolated somata. To examine whether isolated somata of mormyrid Purkinje cells share this ability to support complex spike firing, we have used the dynamic clamp technique to apply complex spikes as command potentials to dissociated rat and mormyrid Purkinje cells (Fig.8). Remarkably, the ability to activate Na$^{+}$ current transients in response to individual spikelets present in the complex spike waveform can also be found in mormyrid Purkinje cells. This observation indicates that there are no qualitative differences in electrophysiological parameters between isolated somata of rat and mormyrid Purkinje cells. These findings add weight to the hypothesis that the absence of synaptically evoked complex spikes in mormyrid Purkinje cells is due to synaptic/dendritic integration properties, which might be based on morphological differences. However, no direct evidence is available yet to support this idea.
and thus other factors need to be considered as well. For example, differences in response
amplitudes could have an impact on response characteristics. The observed difference in the
CF EPSC amplitude (Fig. 1D) could, for example, play a role in the different response
patterns. Our recordings of CF responses of different amplitudes and at different membrane
potentials allow us, however, to exclude the possibility that this phenomenon is solely related
to the spike threshold. Remarkably, a larger amplitude was also observed when comparing the
fast, inactivating Na⁺ current measured in rat Purkinje cells in slices to those recorded in fish
Purkinje cells (Fig. 7). A similar amplitude difference in the initial spike component can be
seen in the dynamic clamp recording shown in Fig. 8. The hypothesis that a lower Na⁺ current
amplitude explains the absence of complex spikes in fish Purkinje cells is, however, not
supported by the data that are best suited for a quantitative comparison: we neither detected
significant differences in the amplitudes of the fast, inactivating Na⁺ current, nor the resurgent
Na⁺ current in dissociated Purkinje cells (Fig. 6).

Interestingly, the initial complex spike component, attributed to a somatic Na⁺ spike, is
completely absent in mormyrid Purkinje cells. Na⁺ spikes can be recorded in these neurons,
but they do not reach the amplitudes seen in other types of neurons and typically stay below
30mV. As outlined above, we could not detect significant differences in Na⁺ current
amplitudes in the dissociated cell configuration. Therefore, we consider it more likely that the
difference in Na⁺ spike amplitudes has morphological causes. For example, the thin,
unmyelinated axons of mormyrid Purkinje cells might reduce axonal spike propagation from
the axonal spike initiation zone, which has been shown in rat Purkinje cells to be at the first
node of Ranvier (Clark et al., 2005), towards the soma (see also Han and Bell, 2003).
Purkinje cells of mormyrid fish are very interesting for reasons that go beyond the described
differences in synaptic responses and in Na⁺ spike activity. They are, for example, optimally
suited to study the impact of dendritic tree architecture (e.g. degree of dendritic branching) on
dendritic integration and spike propagation (see Vetter et al., 2001). Further studies on the expression profiles of voltage-dependent ion channels will be necessary to better understand what electrophysiological or morphological parameters critically contribute to the unique response features of mormyrid Purkinje cells.
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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PF(s)</td>
<td>Parallel Fiber(s)</td>
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<tr>
<td>CF</td>
<td>Climbing Fiber</td>
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<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
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<tr>
<td>PB</td>
<td>Phosphate Buffer</td>
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<td>TBS</td>
<td>Tris Buffered Saline</td>
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<td>ABC</td>
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<td>Flourescein-isotiocyanate</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>LSM</td>
<td>Laser Scanning Microscope</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial Cerebrospinal Fluid</td>
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<tr>
<td>rACSF</td>
<td>Resurgent Artificial Cerebrospinal Fluid</td>
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<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory Post Synaptic Current</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol Phosphate 3</td>
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**Hansel C, and Linden DJ.** Long-Term Depression of the Cerebellar Climbing Fiber


Figure legends

Fig. 1. Climbing fiber-evoked electrical responses in Purkinje cells. A: Complex spike recorded from a rat Purkinje cell. B: All-or-none climbing fiber responses recorded from a *Gnathonemus petersii* Purkinje cell. Responses are shown above and below the threshold for evoking an EPSP. The inset shows a series of parallel fiber EPSPs evoked at increasing stimulus intensities. Note the different time scales of the CF and PF response. C: Example of a cell in which the EPSP was followed by small spikes. D: Voltage dependence of CF EPSCs recorded from rat (left) and mormyrid Purkinje cells (right). Voltage steps were +30, +20, +15, +10, 0 and −10 (in mV) for the rat recording and +30, +20, +10, 0 and −10 (in mV) for the fish recording. E: Voltage dependence of CF-EPSCs measured in rat (n=5) and fish Purkinje cells (n=4). Not every voltage step was applied to every cell. Amplitudes are shown as mean ± SEM. F: CF EPSCs from Fig. 1D show comparable rising phase kinetics above and below the reversal potential (the positive currents were reversed and scaled to the same amplitude). Top: Currents recorded from a rat Purkinje cell at +30 and 0mV. Bottom: Currents recorded from a fish Purkinje cell at +30 and −10mV. Current amplitudes differ for the traces shown and amplitude scale bars were therefore omitted. The original traces are shown in Fig. 1D.

Fig. 2. IP$_3$-receptor subtype I antibody staining A: Rat cerebellar section stained with anti-IP$_3$-receptor subtype I antibodies and DAB. Note the Purkinje cell specificity of the IP$_3$-receptor in the cerebellum and the evenly dense staining in the somata and dendrites of the Purkinje cells. Top left a few Purkinje cell axons are stained. B: Staining as in panel A, but in a section of the mormyrid cerebellum. Note that the staining pattern of IP$_3$-receptors is very similar between rat and fish (panels A and B), but that the architecture of the Purkinje cell dendrite is
different. C: Mormyrid cerebellar section stained with anti-IP$_3$-receptor subtype I antibodies and FITC. Note that the dendrites of mormyrid Purkinje cells branch only a few times very proximally and then run straight to the pial surface. D: The dendritic tree of a mormyrid Purkinje cell clearly shows numerous spines that are stained for IP$_3$-receptors of subtype I (FITC). Scale bars = 12.5 µm in A+B, 20 µm in C and 2µm in D.

Fig. 3. Na$_{v}$1.1 channel staining. A: Anti-Na$_{v}$1.1 channel antibody and DAB staining. Note the denser staining of Na$_{v}$1.1 channels in the somata of the rat Purkinje cells versus the weaker staining in the dendrites. B: Higher magnification of panel A. C: Anti-Na$_{v}$1.1 channel antibody staining of somata in the ganglion cell layer of mormyrid Purkinje cells. Note the comparable staining pattern between panels A and C. Also note the staining of cells in the granule cell layer (bottom right). D: Higher magnification of panel C. Scale bars = 25 µm in A+C and 6 µm in B+D. E: Preadsorption control for anti-Na$_{v}$1.1 antibody on a mormyrid cerebellar section. F: Omission of primary antibody shows weak unspecific binding of secondary antibody comparable to that in panel E. Scale bar = 25 µm in E and 6 µm in F. G: Western blot analysis shows that anti- Na$_{v}$1.1 antibodies bind to proteins of the appropriate size in both fish and rat brain membrane preparations.

Fig. 4. Na$_{v}$1.2 channel staining. A: Anti-Na$_{v}$1.2 channel antibody and DAB staining. Na$_{v}$1.2 staining is equally dense in the somata and dendrites of rat Purkinje cells. B: Higher magnification of panel A. C: Same staining as in panel A but now of mormyrid Purkinje cells. Note that the density of the Na$_{v}$1.2 staining is comparable between rat and fish Purkinje cells. D: Higher magnification of panel C. Scale bars = 25 µm in A+C and 6 µm in B+D. E: Preadsorption control for anti-Na$_{v}$1.2 antibody on a mormyrid cerebellar section. F: Omission of primary antibody shows weak unspecific binding of secondary antibody comparable to that
in panel E. Scale bars = 25 µm in E+F. **G:** Western blot analysis shows that anti- Na\(_{\text{v}}\)1.2 antibodies bind to proteins of the appropriate size in both fish and rat brain membrane preparations.

Fig. 5. Na\(_{\text{v}}\)1.6 channel staining. **A:** Anti-Na\(_{\text{v}}\)1.6 channel antibody and DAB staining. Na\(_{\text{v}}\)1.6 staining is in the rat present in the somata and in the dendrites. **B:** Higher magnification of panel A. **C:** Same staining as in panel A but now of mormyrid Purkinje cells. Note that the density of the Na\(_{\text{v}}\)1.6 staining is comparable between rat and fish Purkinje cells. **D:** Higher magnification of panel C. Scale bars = 25 µm in A+C and 6 µm in B+D. **E:** Preabsorption control for anti-Na\(_{\text{v}}\)1.6 antibody on a mormyrid cerebellar section. **F:** Omission of primary antibody shows weak unspecific binding of secondary antibody comparable to that in panel E. Scale bars = 25 µm in E+F. **G:** Western blot analysis shows that anti- Na\(_{\text{v}}\)1.6 antibodies bind to proteins of the appropriate size in both fish and rat brain membrane preparations.

Fig. 6. Fast inactivating and resurgent Na\(^+\) currents recorded from dissociated rat and mormyrid fish Purkinje neurons. **A:** Voltage step protocol used to elicit Na\(^+\) currents. **B:** TTX subtracted fast inactivating (+30 mV step) and resurgent Na\(^+\) currents (-60 to –10 mV steps) recorded from rat dissociated Purkinje neurons. **C:** TTX subtracted fast inactivating and resurgent Na\(^+\) currents recorded from mormyrid dissociated Purkinje neurons. **D:** Voltage-step protocols were applied to determine the input resistances of rat (left) and fish (right) Purkinje cells. **E:** Enlargement of peak resurgent Na\(^+\) currents recorded **F:** Fast Na\(^+\) currents recorded during wash-in of TTX. **G:** Current-voltage relationship of resurgent Na\(^+\) currents. **H:** Peak fast Na\(^+\) current. **I:** Rise time of resurgent Na\(^+\) currents. **J:** Rise time of fast Na\(^+\)
current. **K:** 50% decay time of resurgent Na⁺ currents. Measured from peak to 50% decay of the peak current. **L:** 50% decay time of fast Na⁺ current. Panels G through L show the average +/- SEM, rat n=5, mormyrid n=6.

Fig. 7. Fast inactivating and resurgent Na⁺ currents recorded from rat and mormyrid fish cerebellar Purkinje neurons in slices. **A:** Voltage step and voltage ramp protocols used to elicit Na⁺ currents. Note the different time scales. **B:** Three consecutive TTX subtracted fast Na⁺ current traces recorded 20 seconds apart during voltage steps from –90 to +30 mV from a rat Purkinje cell. **C:** TTX subtracted resurgent Na⁺ current recorded from a rat Purkinje cell during a step from +30 to –30 mV. **D:** Same as in B but from mormyrid Purkinje cell. **E:** Same as in C but from mormyrid Purkinje cell. **F:** Peak fast Na⁺ current recorded from rat (average +/- SEM, n=3) and mormyrid (average +/- SEM, n=5) Purkinje cells in slices. **G:** Fast Na⁺ current kinetics recorded from rat (average +/- SEM, n=3) and mormyrid (average +/- SEM, n=5) Purkinje cells in slices. **H:** Peak resurgent Na⁺ current recorded from rat (average +/- SEM, n=7) and mormyrid (average +/- SEM, n=6) Purkinje cells in slices.

Fig. 8. Na⁺ currents recorded in dynamic clamp configuration in response to a complex spike applied as a command potential. **A:** Trace on top shows the command waveform that was applied to a dissociated rat Purkinje cell. The complex spike was recorded in a cerebellar slice from an intact Purkinje cell in current-clamp mode. The bottom trace shows the Na⁺ current (TTX subtracted) recorded from a dissociated Purkinje cell in voltage-clamp mode. **B:** The same command waveform was applied to a dissociated mormyrid Purkinje cell in voltage-clamp mode. The bottom trace trace shows the Na⁺ current (TTX subtracted). Note the different current amplitude scale in (B).
Figure 1:
Figure 2:
Figure 3:
Figure 4:
Figure 5:
Figure 6:
Figure 7:

A. Diagram showing voltage steps.

B. Graph showing current response with 1 pA scale.

C. Graph showing current response with 100 pA scale.

D. Graph showing current response with 400 pA scale.

E. Graph showing current response with 200 pA scale.

F. Bar graph comparing fast sodium current peak between Rat and Mommyrd.

G. Bar graph comparing fast sodium current kinetics.

H. Bar graph comparing resurgent sodium current peak between Rat and Mommyrd.
Figure 8: