Title	Development of an anatomical technique for visualizing the mode of climbing fiber innervation in Purkinje cells and its application to mutant mice lacking GluR 2 and Cav2.1
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Citation	Anatomical Science International, 86(1), 10-18 https://doi.org/10.1007/s12565-010-0095-1
Issue Date	2011-03
Doc URL	http://hdl.handle.net/2115/47272
Rights	The original publication is available at www.springerlink.com
Type	article (author version)
File Information	ASI86-1_10-18.pdf

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Development of anatomical technique visualizing the mode of climbing fiber innervation in Purkinje cells and its application to mutant mice lacking GluR δ 2 and Ca v 2.1

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Short running title: Tracer/VGluT2 labeling of CF innervation

24 Text Pages, 5 Figures,

Number of words: Abstract, 235

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Key Words (Five words): Purkinje cell, climbing fiber, cerebellum, anterograde tracer labeling, type 2 vesicular glutamate transporter

Acknowledgments

This study was supported through Special Coordination Funds for promoting Science and Technology, Grants-in-Aid for Scientific Research (19100005 to MW) provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Abstract

In the adult cerebellum, a single climbing fiber (CF) innervates proximal dendrites of Purkinje cells (PCs). This mono-innervation is established by developmental elimination of surplus CFs through homosynaptic competition among multiply-innervating CFs and heterosynaptic competition between CFs and parallel fibers, i.e., granule cell axons innervating distal PC dendrites. Although developmental process of CF mono-innervation and its defects in mutant and experimental animal models have been extensively studied by electrophysiological techniques, relevant morphological information had been poorly understood, because of the lack of neuroanatomical methods to distinguish CFs of different neuronal origins. Soon after the identification of type 2 vesicular glutamate transporter (VGluT2) that selectively detects CF terminals in the molecular layer, we developed a novel method of combined anterograde tracer labeling and VGluT2 immunohistochemistry. This method enables us to identify the mode (mono-innervation vs. multiple innervation) of CF innervation and the site of multiple innervation. Since then, we have applied this method to various kinds of gene-manipulated mice manifesting ataxia and other cerebellar phenotypes. In this review, we summarize experimental procedures for the combined tracer/VGluT2 labeling method, and then introduce what we learned with this method when applied to studies on the role of GluR δ 2 and Ca_v2.1 in CF mono-innervation. This method has provided informative anatomical correlates to electrophysiological data and vice versa, and will extend our knowledge on the molecular and cellular mechanisms for development, plasticity, degeneration, and repair of the CF-PC projection system.

Introduction

Cerebellar Purkinje cells (PCs) are innervated by two excitatory afferents: climbing fibers (CFs) originating from the inferior olivary nucleus and parallel fibers (PFs) of cerebellar granule cells (Palay and Chan-Palay, 1974). Each PC is innervated by $10^5 \sim 10^6$ of PFs at distal dendrites (also called spiny branchlets), while shaft or proximal dendrites are innervated by a single CF but it forms hundreds of synapses on to the innervating PCs. Accordingly, CF activity can cause strong depolarization and trigger Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs) (Kano et al., 1992; Konnerth et al., 1992; Regehr and Mintz, 1994). PCs are initially innervated by multiple CFs at birth, and surplus CFs are eliminated one by one until mono-innervation by a single main CF is achieved by the end of the third postnatal week (Hashimoto and Kano, 2003; Hashimoto et al., 2009). However, innervation by multiple CFs persists into adulthood in hypogranular animal models, where PF synaptogenesis is impaired by x-ray irradiation or spontaneous gene mutation (Woodward et al., 1974; Crepel et al., 1981; Mariani, 1982; Bravin et al., 1995; Sugihara et al., 2000). These lines of experimental evidence indicate that CF mono-innervation is established by homosynaptic competition among multiple CFs and heterosynaptic competition between PFs and CFs.

Analyses using gene-manipulated mice have extended our understanding that signaling molecules expressed at PC synapses regulate PF synaptogenesis and elimination of surplus CFs, both being the essential step for CF mono-innervation. These synaptic molecules include the metabotropic glutamate receptor mGluR1 - Gq protein subunit $\text{G}\alpha_q$ - phospholipase $\text{C}\beta_4$ -

protein kinase γ - pathway, the α 1A subunit of P/Q-type Ca^{2+} channels ($\text{Ca}_v2.1$), GluR δ 2, and Cbln1 (Kano et al., 1995, 1997, 1998; Kashiwabuchi et al., 1995; Offermanns et al., 1997; Ichikawa et al., 2002; Miyazaki et al., 2004; Hirai et al., 2005b). In most of these analyses, the persistence of multiple CF innervation has been determined by whole-cell recoding, which can detect the step number of CF-evoked excitatory postsynaptic currents (EPSCs) in a given PC. The sites of innervation by main and surplus CFs were further estimated by comparing the waveform of CF-EPSCs, such as the amplitude, rise time, and decay time. In the course of these studies, electrophysiological techniques solved many questions, leaving most anatomical questions unanswered. Especially, a novel neuroanatomical method visualizing the mode of CF innervation was necessary for discussing with electrophysiological findings and specifying molecular roles more in detail for the process of CF mono-innervation.

Ten years ago, type 2 vesicular glutamate transporter (VGluT2) was identified by several groups (Aihara et al., 2000; Fremeau et al., 2001; Herzog et al., 2001). Because VGluT2 is selective to CF terminals in the molecular layer (Fremeau et al., 2001; Miyazaki et al., 2003), we assumed that CFs of different neuronal origins could be distinguished, if immunohistochemical labeling for VGluT2 is combined with anterograde tracer labeling of CFs. In this review, we first describe the combined tracer/VGluT2 labeling method to visualize the mode of CF innervation, and then introduce what we have obtained with this method from our experience of its application to mutant mice lacking GluR δ 2 and $\text{Ca}_v2.1$. As to functional and molecular aspects of these molecules, see other reviews (Watanabe, 2008; Mandolesi et al., 2009; Yuzaki, 2009).

Combined tracer/VGluT2 labeling method

Anterograde tracer labeling of CFs has been widely applied as a classical neuroanatomical method to investigate the trajectory of single axons from the inferior olive to the cerebellum, mediolateral and rostrocaudal distribution of CFs in the cerebellar cortex, and branching and terminal bouton formation of CFs in the molecular layer (Sugihara et al., 1999, 2001). We employed the dorsal approach to inject anterograde tracers into the inferior olive, and combined this with VGluT2 immunohistochemical labeling.

For tracer injection, we use stereotaxic instrument (SR-5N, Narishige, Fig. 1A) and glass pipettes prepared by a puller (PC-10, Narishige). A glass pipette, filled with 2-3 μ L of 10% solution of biotinylated dextran amine (BDA; 3,000 molecular weight; Invitrogen) for bright-field light microscopy and electron microscopy or dextran Alexa 594 (DA-594, Invitrogen) for confocal laser scanning microscopy in phosphate-buffered saline (pH 7.4), is connected with a silicone tube to a picopump (Pneumatic Picopump; World Precision Instruments, Sarasota, FL) and set to a micromanipulator (Fig. 1A, B). Under deep anesthesia by chloral hydrate (350 mg/kg of body weight, i.p.), a mouse is clamped by ear bars at external acoustic foramen, and the line between the clamping point and the maxilla is set parallel to the horizontal plane (Fig. 1C, F). After confirming no responses of anesthetized mice to tactile stimulation, the dorsal medulla is exposed by surgical opening of the posterior atlanto-occipital membrane (Fig. 1D, E). In adult mice (about 20 g of body weight), the glass pipette is inserted into the medulla at an angle of 58° to 59° to the perpendicular line, 1.00 mm

lateral from the midline, and 1.70 to 1.85 mm deep from the surface of the medulla (Fig. 1E, F). Tracer is injected by air pressure at 20 psi with 5 s intervals for 1 min, and the surgical cut is closed using instant adhesive, Aron Alpha A Sankyo (Toagosei Co., Ltd., Tokyo, Japan). After 4 days of survival, mice are transcardially perfused with 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) for light microscopy or with the fixative containing 0.1% glutaraldehyde for electron microscopy. After sectioning on a microslicer (50 μ m in thickness), BDA is visualized by overnight incubation with peroxidase-labeled streptavidin (Nichirei, Tokyo, Japan) and colored in black using 3,3'-diaminobenzidine (DAB) and cobalt. Successful injection, which can be confirmed in coronal sections through the inferior olivary nucleus (Fig. 1G), yields selective axon labeling running in the white matter and granular layer of the cerebellum (Fig. 1H) and exhibits typical branching and varicosities of CFs in the molecular layer (Fig. 1I).

Fig. 2A and 2B show images by the confocal laser scanning microscopy of parasagittal cerebellar sections labeled for fluorescent tracer DA-594, VGluT2, and calbindin. Because DA-594 labels a limited number of CFs, the course and branching of a given labeled CF can be traced in the molecular layer (Fig. 2A₁). When combined with immunofluorescence for calbindin, a marker molecule of PCs, DA594-labeled CFs are visualized to climb up the molecular layer by tightly associating shaft dendrites of PCs (Fig. 2A₂). When further applying VGluT2 immunofluorescence that labels theoretically all CF terminals within the extent of antibody-accessible depths in sections, dually labeled (tracer-labeled/VGluT2-labeled) CFs (dCF) are clearly distinguished from singly

labeled (tracer-unlabeled/VGluT2-labeled) CF terminals (sCF) (Fig. 2A₃). Importantly, dCF and sCF, which thereby originate from different inferior olivary neurons, are wired to shaft dendrites of different PCs, and never intermingled at the same dendrites in wild-type mice (Fig. 2B). At the electron microscopic level, dCF and sCF can be distinguished by dual labeling by immunogold for VGluT2 and immunoperoxidase for BDA. Fig. 2C-E show two shaft dendrites, PCD-a and PCD-b. Spines protruding from PCD-a are all innervated by dCF (Fig. 2C, D), while those from PCD-b are all innervated by sCF (Fig. 2C, E).

These results indicate that CFs of different neuronal origins can be anatomically distinguished by the combined tracer/VGluT2 labeling method at both the light and electron microscopic levels. Since then, we have been applying this method to analyses of various gene-knockout (KO) mice to address the mode of CF innervation, i.e., mono-innervation vs. multiple innervation, and the site of additional innervation by surplus CFs (Fig. 2F) (Miyazaki et al., 2004, 2006, 2010; Kakizawa et al., 2005; Tohgo et al., 2006; Ikeda et al., 2007; Uemura et al., 2007; Tomioka et al., 2008; Watanabe et al., 2008). Of these, we have most intensively analyzed the cerebella of GluR δ 2-KO and Ca_v2.1-KO mice, both being known to manifest severe persistence of multiple CF innervation electrophysiologically and suffer from severe ataxia behaviorally (Hashimoto et al., 2001; Ichikawa et al., 2002; Miyazaki et al., 2004).

Application to GluR δ 2-KO mice

Although GluR δ 2 is a member of 18 subunits of ionotropic glutamate receptors (Araki et al., 1993; Lomeli et al., 1993), it does not function as glutamate-gated

ion channels (Hirai et al., 2005a; Kakegawa et al., 2007a, b). Rather, GluR δ 2 plays an important role in the formation and maintenance of the PF-PC synapse (Guastavino et al., 1990; Kashiwabuchi et al., 1995; Kurihara et al., 1997; Lalouette et al., 2001; Takeuchi et al., 2005) through its selective expression at this synapse (Araki et al., 1993; Lomeli et al., 1993; Takayama et al., 1995; Landsend et al., 1997) and specific interaction of its *N*-terminal domain with presynaptic molecule neurexin through Cbln1 released from granule cells (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Lalouette et al., 2001; Yuzaki, 2009; Matsuda et al., 2010; Uemura et al., 2010). Primary defects in GluR δ 2-KO mice occur in the connectivity of PF-PC synapses, as manifested by the emergence of free spines and mismatching of pre- and postsynaptic specialization (Guastavino et al., 1990; Kurihara et al., 1997; Lalouette et al., 2001). Free spines are most numerous at the distal dendritic domain innervated by PFs only and also found at the intermediate domain receiving mixed PF and CF innervation, whereas the proximal domain only innervated by CFs lacks free spines (Ichikawa et al., 2002). This suggests that free spines are generated by failed synaptic contact with PF terminals. Moreover, the loss of GluR δ 2 causes the persistence of multiple innervation (Kashiwabuchi et al., 1995; Hashimoto et al., 2001; Ichikawa et al., 2002).

The combined tracer/VGluT2 labeling method visualized that both dCF and sCF are distributed in basal four-fifths of the molecular layer in wild-type mice (Fig. 2A), whereas they expand their territory almost up to the pial surface in GluR δ 2-KO mice (Ichikawa et al., 2002; Miyazaki et al., 2010; Fig. 3A, C). This expansion of CF territory occurs due to distal extension of CFs to innervate free

spines generated at the distal and intermediate dendritic domains (Ichikawa et al., 2002; Miyazaki et al., 2004). As climbing up along dendrites, CFs in GluR δ 2-KO mice often dissociate from the host dendrites and jump to adjacent dendrites (arrowheads in Fig. 3B). In such portions, the same dendrites are multiply innervated by dCF and sCF (red and green arrows in Fig. 3B). In addition, dCF innervating a small portion of distal dendrites appears abruptly in the parasagittal plane (Fig. 3C). In such cases, ectopic innervation by dCF causes multiple innervation together with sCF (Fig. 3D). Therefore, this method can clearly demonstrate that aberrant jumping and wiring of CFs on to adjacent dendrites result in multiple innervation in GluR δ 2-KO mice.

In addition to the main ascending branch of CFs, there is another branch termed the transverse branch. This branch is a thin motile collateral, which extends transversely (or mediolaterally) and rarely forms conventional synapses in wild-type rodents (Rossi et al., 1991; Sugihara et al., 1999; Nishiyama et al., 2007). This branch is well identified and traced when using transverse cerebellar sections. On transverse sections, PC dendrites are seen as single straight *bars*, main ascending branches as *ladders* on the dendritic bars, and transverse branches as thin horizontal fibers emitting from the ladders (Fig. 4, 5A). The combined tracer/VGluT2 labeling method is also effective in grasping phenotypic alterations of transverse branches (Miyazaki et al., 2010). In control mice, transverse branches rarely differentiate VGluT2-positive terminals (arrowheads in Fig. 4B). In GluR δ 2-KO mice, however, they are substantially elongated in the mediolateral extent, frequently form VGluT2-positive terminals, and innervate dendritic bars of nearby and remote PCs (red arrows in Fig. 4D). Because the

rest of dendritic bars are mostly innervated by sCF (green arrows), aberrant wiring by transverse CF branches also causes multiple innervation in GluR δ 2-KO mice.

Therefore, this method successfully visualizes that the ablation of GluR δ 2 permits aberrant wiring to PC dendrites by the ascending and transverse branches of CFs. Notably, aberrant wiring causing multiple CF innervation often occurs against distal dendrites, as indicated from distal extension of CF territory (Fig. 3A, C), aberrant jumping to distal dendrites (Fig. 3B), and aberrant elongation of synapse-forming transverse branches in superficial portions of the molecular layer (Fig. 4C, D). These phenotypes collectively suggest that the primary role of GluR δ 2 is to ensure PF-PC synapse formation at distal dendrites, which eventually restricts CF innervation to proximal dendrites and suppresses multiple CF innervation at distal dendrites (Fig. 5B, left). This notion from anatomical analyses is consistent with an electrophysiological study showing that stimulation of the main CF elicits large EPSCs with a fast rise time and high Ca^{2+} elevation spreading throughout the dendritic tree, whereas that of surplus CFs elicit small EPSCs with a slow rise time and low Ca^{2+} elevation confined to local distal dendrites (Hashimoto et al., 2001).

Application to $\text{Ca}_v2.1$ mice

Aforementioned phenotypes suggest that the two inputs to PCs are highly competitive to each other, with one input expanding its own territory at the expense of the other. If GluR δ 2 regulates this competition to the advantage of PFs, there should be the counter mechanisms that expand the CF territory at the

expense of the PF territory. We assumed that the P/Q-type Ca^{2+} channel is one of the likely candidates, because it mediates > 90% of the total Ca^{2+} current density in PCs (Mintz et al., 1992, 1995; Stea et al., 1994), is high voltage-activated VDCCs requiring strong depolarization, and could play a role as an activity coincidence detector in PCs that express no GluN2 subunits of NMDA receptors (Watanabe et al., 1994; Yamada et al., 2001). To test this hypothesis, we applied the combined tracer/VGluT2 labeling to mutant mice lacking the pore-forming subunit or $\text{Ca}_v2.1$ (Miyazaki et al., 2004).

In $\text{Ca}_v2.1$ -KO mice, the territory of CF innervation is regressed to a basal half of the molecular layer (Fig. 3E). In most PCs, both dCF and sCF innervate the soma and proximal dendrites of the same PCs (Fig. 3F-H), indicating high frequency of multiple innervation at the proximal somatodendritic compartment. Conspicuously, surplus CFs (dCF in Fig. 3F and G, sCF in Fig. 3H) also innervate the basal part of proximal dendrites as well as the soma. The somatic innervation by surplus CFs is consistent with electrophysiological data showing fast kinetics in surplus CF-EPSCs (Miyazaki et al., 2004). Furthermore, dendritic translocation of surplus CFs is also parallel with low disparity of amplitudes among multiple CF-EPSCs (Hashimoto et al., 2009). In normal developmental process of CF mono-innervation, only a single main CF translocates to dendrites, whereas surplus CFs remain in the soma and subsequently eliminated (Hashimoto et al., 2009). Therefore, in $\text{Ca}_v2.1$ -KO mice, dendritic translocation of a main CF is weakened, whereas surplus CFs retain somatic innervation and further translocate to dendrites. These characteristic phenotypes suggest that $\text{Ca}_v2.1$ promotes functional differentiation of multiply-innervating CFs into single

main CF and surplus CFs to be eliminated.

Another notable phenotype in $\text{Ca}_v2.1$ -KO mice is reciprocal expansion of the PF territory down to proximal dendrites and somata of PCs (Miyazaki et al., 2004). This is reflected in hyperspiny transformation of somatodendritic domain of PCs and ectopic PF innervation onto these spines. Such hyperspiny transformation and expanded PF innervation are also observed following surgical denervation of CFs (Sotelo et al., 1975) or CF regression following activity blockade by TTX (Bravin et al., 1999; Morando et al., 2001; Cesa et al., 2003, 2007). Thus, $\text{Ca}_v2.1$ potently suppresses proximal expansion of the PF territory. $\text{Ca}_v2.1$ therefore fuels both homosynaptic competition among multiple CFs and heterosynaptic competition between PFs and CFs to the advantage of the latter (Fig. 5B, right). Therefore, armed with both $\text{GluR}\delta 2$ and $\text{Ca}_v2.1$, highly territorial patterns of PF and CF innervation are normally structured along PC dendrites, and surplus CFs are safely eliminated from distal and proximal dendrites, leading to CF mono-innervation (Fig. 5B, middle).

In conclusion, the combined tracer/VGluT2 labeling method provides informative neuroanatomical correlates to electrophysiological data, and vice versa. This will make the CF-PC projection system one of the best circuit models in neuroscience studies on the development, plasticity, degeneration, and repair.

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Figure legends

Figure 1. Anterograde tracer labeling of climbing fibers. (A) An overview of a stereotaxic instrument for tracer injection. (B) A tip of glass pipette filed with tracer solution. (C) A mouse clamped by ear bars and opening of the dorsal medulla (boxed region). (D) An insertion of glass pipette into the medulla. (E) A cartoon indicating the injection point (red cross) of the medulla. A broken line indicates the midline of the medulla. (F) A cartoon indicating the angle of a micromanipulator. Dotted circle indicates clamping point by ear bars. The line between the clamping point and the maxilla is set parallel to the horizontal plane (dotted line). P, perpendicular line. h, horizontal bar of the stereotaxic instrument. (G) Injection site of BDA in a coronal medulla section. BDA is visualized with diaminobenzidine (DAB) in the right inferior olive. (H) A BDA-labeled cerebellar section. BDA is visualized with DAB and cobalt. (I) A typical view of BDA-labeled CF in the molecular layer. Scale bars: G, H, 500 μm ; I, 50 μm .

Figure 2. Combined tracer/VGluT2 labeling method. (A, B) Triple fluorescent labeling for calbindin (A_2 , green, A_3 , blue), VGluT2 (A_3 , green) and anterograde tracer DA-594 (A_1 , red). In B, two dendrites PCD-a and PCD-b are enlarged from a boxed region of A. PCD-a is innervated by dually labeled (tracer-labeled/VGluT2-labeled) CFs (dCF), while PCD-b is innervated by singly labeled (tracer-unlabeled/VGluT2-labeled) CFs (sCF). (C-E) Double immunoelectron microscopy for BDA (diffuse DAB precipitates) and VGluT2 (metal particles). PCD-a (pseudocolored in brown) is innervated by dually labeled (tracer-labeled/VGluT2-labeled) CF (dCF), while PCD-b (green) is

innervated by singly labeled (tracer-unlabeled/VGluT2-labeled) CF (sCF). Asterisks indicate two spines, which protrude from PCD-a or PCD-b (arrowheads), respectively and innervated by dCF or sCF, respectively. Boxed regions in C are enlarged in D and E. (F) A Cartoon showing anatomical patterns of CF mono-innervation (upper) and multiple innervation (lower) as judging from the combined tracer/VGluT2 labeling method. Scale bars, A, 20 μm ; B, 10 μm ; C, 1 μm ; D, E, 500 nm.

Figure 3. Combined tracer/VGluT2 labeling method applied to parasagittal sections of GluR δ 2-knockout (KO, A-D) and Ca $_v$ 2.1-KO (E-H) mice. All images are subjected to triple fluorescent labeling for calbindin (blue), VGluT2 (green) and DA-594. Boxed regions in A, C, and E are enlarged or separated in B, D and F. Arrowheads in B indicate a tracer-labeled/VGluT2-labeled dCF, which jumps to the adjacent dendrite. Red or green arrows indicate CF terminals of DA-594-labeled dCF or DA-594-unlabeled sCFs, respectively. Scale bars, A, C, E, 20 μm ; B, D, F, G, H, 10 μm .

Figure 4. Combined tracer/VGluT2 labeling method applied to transverse sections of control (A, B) and GluR δ 2-KO (C, D) mice. Triple fluorescent labeling (see a legend of Figure 3). Boxed regions in A and C are enlarged in B and D, respectively. Arrows indicate transverse branches of CFs. Arrowheads indicate VGluT2-negative transverse branches in control mice. Red arrows in D indicate DA-594-labeled transverse branches (dCF) in GluR δ 2-KO mice, which carry VGluT2-positive terminals and wire to distal dendrites innervated by

DA-594-unlabeled sCFs (green arrows). Scale bars, A, C, 20 μm ; B, D, E, 10 μm .

Figure 5. Schematic drawings illustrating phenotypic changes in the mode of CF innervation in PCs. (A) CF-PC innervation in control (upper) and GluR δ 2-KO mice (lower) in the parasagittal (left) and transverse plane (right). In control mice, ascending axons of CFs keeps mono-innervation to the innervating PC, while transverse branches show no synapse formation to nearby PCs. In GluR δ 2-KO mice, both ascending axons and transverse branches exhibit aberrant wiring to nearby and remote PCs, causing multiple CF innervation. (B) Reciprocal phenotypes between GluR δ 2-KO and Ca $_v$ 2.1-KO mice. In GluR δ 2-KO mice, innervation by PF at distal dendrites is impaired, whereas CF innervation extends distally and causes multiple CF innervation at distal dendrites. In Ca $_v$ 2.1-KO mice, on the contrary, PF territory expands down to proximal dendrites and somata, whereas CF innervation regresses proximally and causes multiple CF innervation at the proximal somatodendritic compartment. This figure is modified from Watanabe (2008).

Miyazaki et al., Fig.1

Miyazaki et al., Fig.2

GluR δ 2-KO

Cav2.1-KO

Miyazaki et al., Fig. 4

Miyazaki et al., Fig. 5