Proteomic Studies of a Single CNS Synapse Type: The Parallel Fiber/ Purkinje Cell Synapse

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Introduction

Each of the thousands of cell types present in the nervous system receives multiple classes of inputs that are spatially segregated and functionally distinct. The chemoaffinity hypothesis stated in part that "the establishment and maintenance of synaptic associations were conceived to be regulated by highly specific cytochemical affinities" (Sperry, 1963). Support for this idea has come from studies of specific synaptic proteins (Benson et al., 2001) and from physiological studies of synaptic plasticity (Jorntell and Hansel, 2006). More than 1000 different postsynaptic proteins have been identified in "bulk" postsynaptic density preparations or in affinity-purified receptor complexes (Cho et al., 1992; Husi et al., 2000; Walikonis et al., 2000; Peng et al., 2004; Cheng et al., 2006; Collins et al., 2006). However, studies aimed at systematically identifying the protein composition of individual synapse types, and understanding their mechanistic diversity, have not been reported. To address this gap, we have developed synaptic protein profiling as an approach to isolate and biochemically characterize specific types of CNS synapses. We chose to first analyze the parallel fiber to Purkinje cell (PF/PC) synapse in the cerebellum because of its unique physiological properties and its involvement in neurological disease (Ito, 2001; Polleux and Lauder, 2004).

Results

To enable purification of PF/PC synapses, we developed a transgenic mouse line that expresses an affinity tag only at the PF/PC synapse. We generated a fusion between the glutamate receptor delta2, GLURδ2, which is specifically localized at the PF/PC postsynaptic density (Yuzaki, 2003), and Venus, a variant of the green fluorescent protein (GFP). The resulting fusion protein, VGLURδ2, is properly processed and transported to the cell surface (data not shown). To express the fusion specifically in

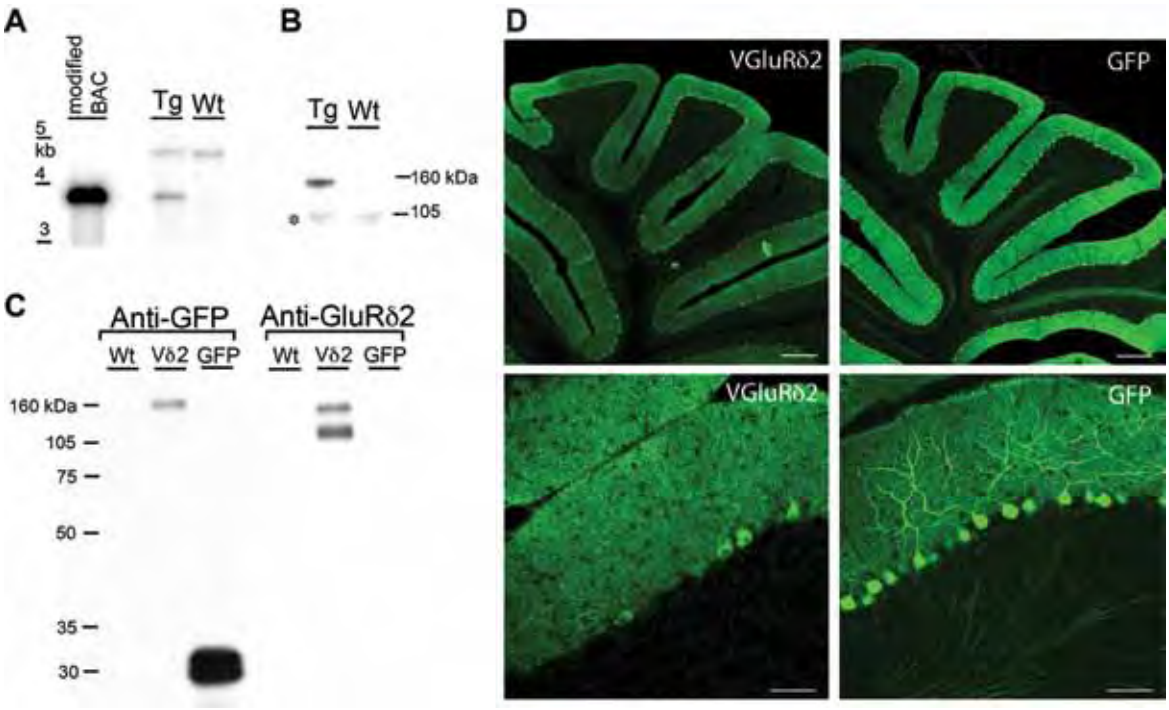

Figure 1. Tagging the PF/PC synapse in transgenic mice. ***A***, Southern blot was used to identify transgenic mice having integrated the *Pcp2* BAC (a Purkinje cell–specific driver) containing the Venus-tagged GLURδ2 receptor, VGLURδ2. ***B***, VGLURδ2 expression was detected using an anti-GFP antibody on immunoblots from total protein extracts of transgenic (Tg) versus wild-type (Wt) cerebella. * indicates a nonspecific band. ***C***, Both VGLURδ2 and GFP were affinity-purified using an anti-GFP antibody from 1% Triton X-100 cerebellar extracts from Wt, *Pcp2/VGluRδ2* (Vδ2), and *Pcp2/eGFP* control (GFP) mice, as shown by probing the immunoblots with an anti-GFP antibody (left). VGLURδ2 specifically copurified the endogenous GLURδ2, as shown by probing the same blot with an anti-GLURδ2 antibody (right). ***D***, Immunofluorescence on cerebellar sections using an anti-GFP antibody shows the specific localization of VGLURδ2 in the molecular layer and somata of Purkinje cells of Pcp2/VGluRδ2 mice. Soluble GFP is detected in the molecular layer, dendrites, somata, and axons of Purkinje cells in sections from Pcp2/eGFP mice. Scale bars: upper panels, 200 μm; lower panels, 50 μm.

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cerebellar Purkinje cells, the VGluRδ2 cDNA was then incorporated into a Pcp2 bacterial artificial chromosome (BAC) by homologous recombination, and the resulting *Pcp2/VGluRδ2* BAC construct was used to generate transgenic mice (Fig. 1*A*).

Expression of the fusion polypeptide was detected in the cerebellar extracts of *Pcp2/VGluRδ2* transgenic mice (Fig. 1*B*), and co-immunoprecipitation experiments demonstrated proper assembly of the VGLURδ2 fusion with the endogenous GLURδ2 receptor subunits (Fig. 1C). As shown in Figure *1D*, the localization of VGLURδ2 in the molecular layer and somata of PCs agrees with the synaptic localization of the GluRδ2 receptor. In contrast, the enhanced green fluorescent protein (eGFP) control protein expressed using the same BAC vector (Pcp2/eGFP) (www.gensat.org) is detected throughout the cell, including marked labeling of both Purkinje cell dendrites and axons (Fig. 1*D*).

Before the affinity purification step, we sought to produce cerebellar extracts enriched for synaptic structures relative to trafficking complexes, and to maximize the recovery of VGLURδ2-tagged postsynaptic densities (PSDs). This was performed by fractionating a solubilized crude synaptosome fraction (S3) on a gel-filtration column (Fig. 2*A*).

As shown in Figure 2*B* and 2C, this biochemical fractionation resulted in an enrichment of synaptic and mitochondrial proteins and a relative depletion of endoplasmic reticulum (ER) components in the high-molecular-weight fractions. These synaptic fractions contain essentially all of the PSD95 scaffolding protein. They also contain VGLURδ2, which was distributed among the different fractions in the same manner as wild-type GluRδ2 (Fig. 2C). This was also observed using standard synaptosome purification (data not shown) and shows that the fusion receptor VGLURδ2 is targeted to the synapse similarly to the wild-type GLURδ2.

To separate PF/PC postsynaptic densities from other cerebellar synapses, we performed affinity purification from the pooled synaptic fractions (red rectangle, Figure 2C) using an anti-eGFP antibody. Electron microscopy of the affinity-purified material showed

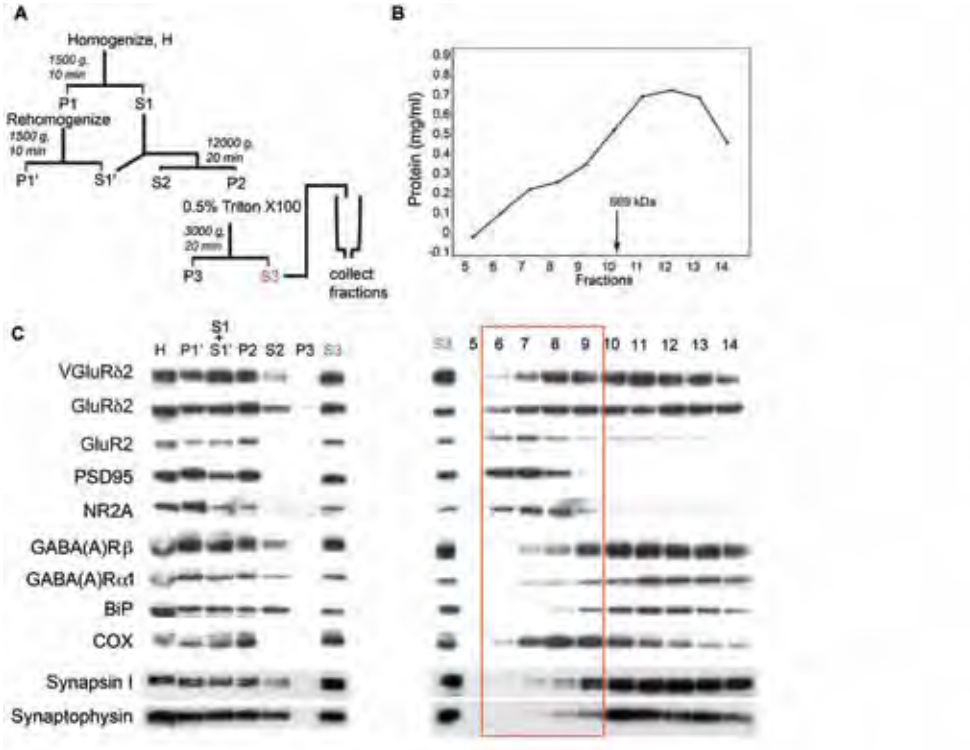

Figure 2. VGLURδ2 is detected in synaptic fractions using a new PSD purification method. ***A***, We prepared a crude synaptosome P2 fraction that was solubilized in 0.5% Triton X-100 final concentration. The extract was then separated on a Sephacryl S1000 gel filtration column (GE Healthcare, Uppsala, Sweden). ***B***, Protein dosage was performed on every fraction collected. ***C***, 0.1% in volume of every fraction was run on Western blots and assayed for the presence of excitatory synapse markers (GLURδ2, GLUR2/3, PSD95, NR2A), inhibitory synapse markers ($GABA_AR\beta$, $GABA_AR\alpha1$), the ER marker BiP, and the mitochondrial marker COX. VGLURδ2 was detected using an anti-GFP antibody. The red rectangle outlines the "synaptic" fractions enriched for synaptic markers and pooled for subsequent affinity purification of PF/PC PSDs.

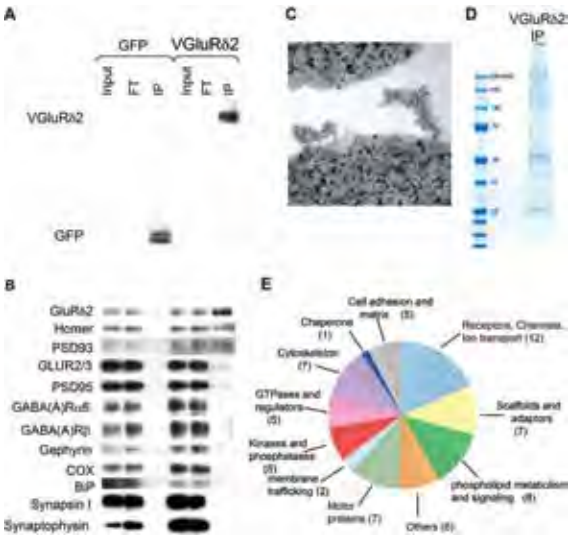

Figure 3. Affinity purification and protein profiling of the PF/PC PSDs. ***A***, Synaptic fractions from *Pcp2/VGluRδ2* animals were affinity-purified using magnetic beads coated with anti-GFP antibody (VGLURδ2). In parallel, control purifications were performed on preparations from *Pcp2/eGFP* transgenic mice (GFP). 0.025% of the inputs and flow-throughs (FT) and 25% of the affinity-purified samples (IP) were assayed by Western blot using an anti-GFP antibody and showed immunoprecipitation of both VGLURδ2 and GFP, respectively. ***B***, The same blot was probed for different synaptic markers and the mitochondrial protein COX, showing specific copurification of synaptic markers localized to the PF/PC synapse. ***C***, Electron microscopy shows the presence of electron-dense structures reminiscent of PSDs on the surface of the magnetic beads used for affinity purification of *Pcp2/VGluRδ2* extracts. ***D***, Mass spectrometry identified 65 different proteins in the complexes purified from *Pcp2/VGluRδ2* mice. ***E***, These proteins can be classified into 11 functional categories. The number of proteins from each category is indicated in parentheses. Nonshaded areas represent proteins found with high confidence.

electron-dense structures that were reminiscent of PSDs (Vinade et al., 2003) on the surface of the beads used for purification of VGLURδ2 extracts (Fig. 3C). These structures were absent from beads used to immunopurify extracts from *Pcp2/eGFP* control cerebella.

Using Western blot analysis, we were able to show that more than 50% of the target protein was immunopurified from the input extract for either the control eGFP or the VGLURδ2 extracts (Fig. 3A and data not shown). Western blotting also demonstrated copurification of several PF/PC synaptic components with VGLURδ2, including the GLURδ2 and GLUR2/3 receptors, and the scaffolding proteins PSD93 and Homer (Fig. 3*B*). Markers of inhibitory synapses ($GABA_A$ receptor α6, $GABA_A$ receptor β, and gephyrin) or of mitochondria (cytochrome oxidase [COX]) did not copurify, demonstrating the specificity of this approach (Fig. 3*B*). As expected, none of these markers copurified with soluble eGFP in extracts prepared from *Pcp2/eGFP* control mice (Fig. 3*B*). Taken together, these results demonstrate that the combination of cell-specific genetic targeting, molecular tagging of specific CNS synapses, biochemical fractionation, and affinity purification can be used to isolate a specific type of PSD from crude brain extracts.

To systematically identify components of the PF/PC PSDs, we analyzed the protein content of pooled PF/PC PSD preparations using single-stage and two-stage mass spectrometry (Cristea et al., 2005). A first experiment, using 30 *Pcp2/VGluRδ2* cerebella, enabled us to identify 12 components present at the PF/PC synapse but not present in the control sample, prepared in parallel from *Pcp2/eGFP* cerebella. To increase the number of PF/PC PSD components identified, we performed a second analysis on a sample prepared from 50 cerebella. A total of 65 proteins was identified: 37 proteins were detected with high confidence, and 28 were observed at lower levels and identified with less confidence. This analysis confirmed the presence of the PF/PC synapse proteins GLURδ2 (Yuzaki, 2003), Homer-3 (Shiraishi et al., 2004), PSD93 (Yuzaki, 2003), delphilin (Yuzaki, 2003), Shank1 and Shank2 (Uemura et al., 2004), and the absence of proteins located at other excitatory (NMDA receptor subunits, $GABA_A$ receptor α6) or inhibitory ($GABA_A$ receptor α6, $GABA_A$ receptor β, and gephyrin) synapses in the cerebellum. Forty of the identified proteins in our affinity-purified PSDs had been previously detected in preparations of synaptic proteins (Collins et al., 2006).

Approximately 700 different proteins have been identified in PSD preparations from whole brain (Collins et al., 2006). However, it has been estimated that, given several factors (the mass of a single PSD, the copy number of scaffold proteins in a PSD, and an average size of 100 kDa for each synaptic protein), only about 100 different proteins can be expected to be found at one particular type of PSD (Sheng and Hoogenraad, 2007). The number of proteins we found in this study was consistent with that estimate. Although it is probable that our analysis failed to identify all PF/PC postsynaptic proteins, the successful identification of AMPA receptor subunits in our preparations suggests that the proteins we failed to identify must be present at low stoichiometries in the PSD.

The 65 proteins we identified can be grouped into 11 different functional categories (Fig. 3*D*). These categories were previously annotated in studies of

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the PSD (Peng et al., 2004), with the exception of a class of proteins that we have called "phospholipid metabolism and signaling." In the "scaffolds and adaptors" category, several members of the Shank family (1 and 2) and the PSD family (PSD93 and PSD95) were detected at the PF/PC synapse, illustrating redundancy for scaffold proteins and underscoring their importance for synaptic function. Other functional categories included proteins important for synapse formation and physiology, such as regulators of small GTPases and protein kinases. Interestingly, eight of the proteins our study identified can regulate or be regulated by phospholipid metabolism (Iptr1, synaptojanin 1 and 2, phospholipase B, ABCA12, MRCKγ) or contain phospholipid-binding domains (Plekha7, annexin A6, MRCKγ). We therefore grouped them into a previously unrecognized category: "phospholipid metabolism and signaling." This suggests that phospholipid regulation is a major feature of the PF/PC synapse, in accordance with the major role that metabotropic glutamate receptor 1 (mGluR1) plays in regulating the physiology of the PF/PC synapse by activating phospholipase C (Ito, 2001; Jorntell and Hansel, 2006).

Another important category present at synapses groups receptors and ion channels: several glutamate receptor subunits and several G protein-coupled receptors ($GABA_B$ and BAI receptors) were detected in our analysis of the PF/PC PSD. Interestingly, the extracellular domain of BAI receptors contains thrombospondin repeats, which can mediate cell adhesion (Adams and Tucker, 2000). Several other proteins identified at the PF/PC synapse in our study are involved in cell adhesion and interaction with the extracellular matrix: receptor protein tyrosine phosphatases (Sallee et al., 2006), delta-catenin-2 (Kosik et al., 2005), Neph1 (Shen and Bargmann, 2003), and laminins (Dityatev and Schachner, 2006). The diversity of these potential recognition proteins at the PF/PC synapse shows the complexity of the "synaptic code" implied by the chemoaffinity hypothesis.

To provide additional evidence for the synaptic localization of the novel components that we have identified, we performed immunofluorescence studies on cerebellar sections from wild-type mice. Localization in the molecular layer of the cerebellum, which contains the PF/PC synapses, was evident for MRCKγ, Gm941, BAIAP2, RPTPm, Neph1, and δ2-catenin (Fig. 4). δ2-catenin and Gm941 could also be detected in some cerebellar

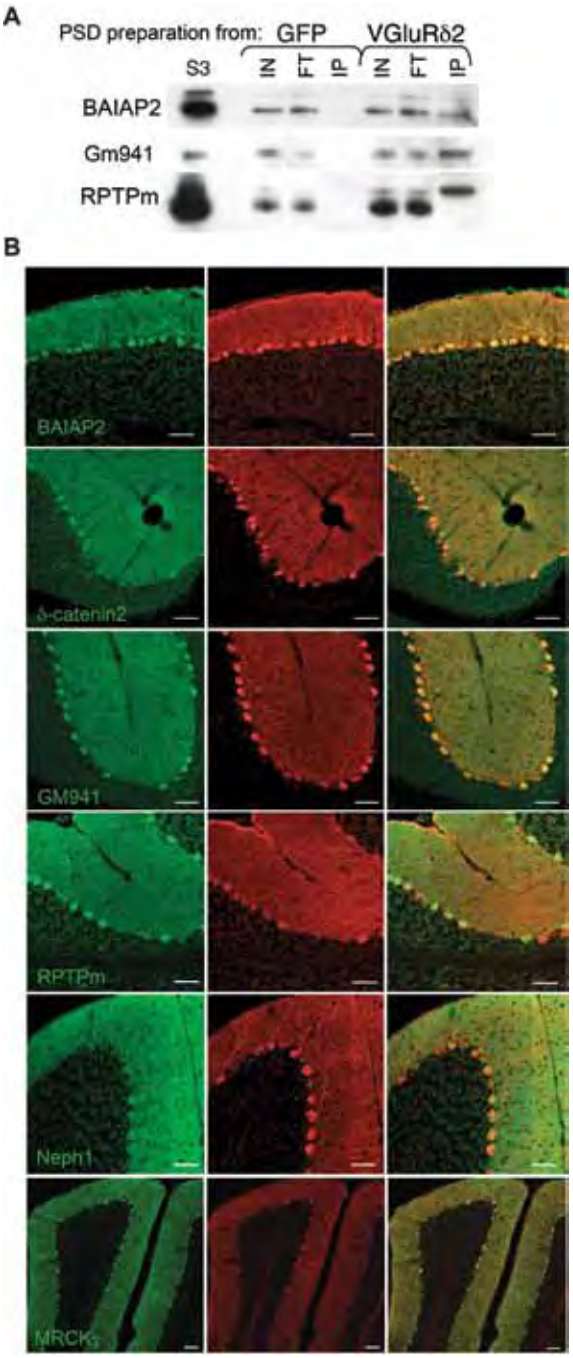

Figure 4. Localization of several candidate synaptic proteins at the PF/PC synapses. ***A***, Presence of selected candidates in PF/PC PSDs purified from *Pcp2/VGluRδ2* cerebella. 0.025% of the inputs (IN) and flow-throughs (FT) and 25% of the affinity-purified samples (IP) obtained from *Pcp2/eGFP* control (GFP) and *Pcp2/ VGluRδ2* (VGluRδ2) cerebella were assayed by Western blot. ***B***, Localization by immunofluorescence of candidate synaptic proteins. Labeling was performed using antibodies recognizing several candidate proteins identified by mass spectrometry (green) in conjunction with an anti-calbindin antibody specifically labeling Purkinje cells (red). Scale bars, 50 μm.

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interneurons. We also examined the expression of candidates reported in *in situ* hybridization databases (www.stjudebgem.org; www.brain-map.org; www.genepaint.org). Interpretable data were available for 36 candidates, and all but two were expressed in Purkinje cells, with a majority showing little detectable expression in the granule cell layer (data not shown). These expression data provide additional confirmation that the majority of the proteins our study identified are bona fide components of the PF/PC synapse.

Discussion

The demonstration that the biochemical components of specific synapse types from a particular neuronal population can be identified using the approach described here has important implications. First, comparative synaptic protein profiling of different CNS synapse types using this strategy (combining specific synaptic tags and genetic targeting) can lead to a direct test of the chemoaffinity hypothesis (Sperry, 1963), as shown by the variety of potential recognition proteins found at the PF/PC synapse in this study. Second, synaptic protein profiling can reveal novel sets of proteins that allow us to formulate specific hypotheses regarding synaptic physiology. For example, the discovery of MRCKγ at PF/PC synapses has implications for the study of neurodevelopmental diseases, such as autism spectrum disorders and mental retardation. There is a frequent occurrence of Purkinje cell aplasia in autism (Polleux and Lauder, 2004) and a link between another small GTPase-dependent kinase, PAK3, and mental retardation (Allen et al., 1998). Third, our results suggest a complex regulatory role for phospholipids at postsynaptic densities, in addition to their important roles in regulating ion-channel permeability, and in vesicle trafficking at the presynaptic membrane (Di Paolo and De Camilli, 2006). We anticipate that further studies of biochemical diversity synapses will be critical for understanding the development and function of specific CNS circuits and their dysfunction in disease (Kennedy, 2000; Serajee et al., 2003).

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