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Climbing Fiber Synapse Elimination in Cerebellar Purkinje Cells

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Abstract

Innervation of Purkinje cells (PCs) by multiple climbing fibers (CFs) is refined into mono-innervation during the first three postnatal weeks of rodents' life. In this review article, we will integrate the current knowledge on developmental process and mechanisms of CF synapse elimination. In the "creeper" stage of CF innervation (postnatal day 0 (P0~), CFs creep among PC somata to form transient synapses on immature dendrites. In the "pericellular nest" stage (P5~), CFs densely surround and innervate PC somata. Then, CF innervation is displaced to the apical portion of PC somata in the "capuchon" stage (P9~), and translocate to dendrites in the "dendritic" (P12~) stage. Along with the developmental changes of CF wiring, functional and morphological distinctions become larger among CF inputs. PCs are initially innervated by >5 CFs with similar strengths (~P3). Only a single CF is selectively strengthened during P3-P7 (functional differentiation), and undergoes dendritic translocation from P9 on (dendritic translocation). Following the functional differentiation, perisomatic CF synapses are eliminated non-selectively, which proceeds in two distinct phases. The early phase (P7-P11) is conducted independently of parallel fiber (PF)-PC synapse formation, while the late phase (P12-P17) critically depends on it. The P/Q-type voltage-dependent Ca^{2+} channel in PCs triggers selective strengthening of single CF inputs, promotes dendritic translocation of the strengthened CFs, and drives the early phase of CF synapse elimination. On the other hand, the late phase is mediated by mGluR1-G α q-PLC β 4-PKC γ signaling cascade in PCs driven at PF-PC synapses, whose structural connectivity is stabilized and maintained by the GluR δ 2-Cbln1-neurexin system.

Introduction

In neural circuit formation, supernumerary synapses are formed transiently around birth. Then, important synapses are functionally strengthened, while unnecessary synapses are weakened and eventually eliminated. This process is known as “synapse elimination” or “synapse pruning”, and is widely accepted as an important step to refine initial redundant circuitry into functionally mature one. The climbing fiber (CF) to Purkinje cell (PC) synapse in the cerebellum provides an excellent model to study the process of synapse elimination in the central nervous system (Crepel 1982; Lohof et al. 1996; Hashimoto and Kano 2005; Kano and Hashimoto 2009).

At two dendritic domains, PCs receive two kinds of excitatory inputs distinct in neuronal origin, wiring diagram, and synaptic strength (Palay and Chan-Palay 1974; Ito 1984). Parallel fibers (PFs) are bifurcated axons of cerebellar granule cells (GCs), and innervate spines on distal dendrites or spiny branchlets. Despite the formation of $10^5\text{-}10^6$ PF synapses in individual PCs, each PF only makes one or two synapses to a given PC (Napper and Harvey, 1988). The strength of individual PF inputs is also weak; the order of 50 simultaneously active GCs are required to generate an action potential, so called “simple spikes” (Palay and Chan-Palay 1974; Ito 1984; Barbour, 1993). In contrast, most PCs in adulthood are mono-innervated by single CFs, but each CF forms 250-300 synaptic contacts along proximal dendritic tree of the innervating PCs (Ito 1984). In addition, CF synapses express five times more AMPA receptors than PF synapses (Masugi-Tokita et al., 2007; Yamasaki et al., 2011). As a result, activation of CFs causes strong depolarization that generates characteristic “complex spikes” (Miyakawa et al., 1992; Eccles et al., 1966; Davie et al., 2008).

An earlier electrophysiological study on juvenile rats *in vivo* shows that

stimulation to the inferior olive after P3 elicits CF-mediated responses in PCs (Crepel, 1971). In contrast to the all-or-none nature of CF responses in adulthood, the responses of juvenile PCs are graded in parallel with the increase of stimulus intensities (Crepel et al., 1976). This is the first evidence that PCs are innervated by multiple CFs in early postnatal development. Subsequent studies *in vivo* revealed that both the percentage of PCs with multiple CF innervation and the average number of CFs innervating individual PCs decrease with postnatal development until attaining the one-to-one relationship (Crepel et al., 1981; Mariani and Changeux, 1981). Reciprocal to the numerical decrease of innervating CFs, summed excitatory postsynaptic currents (EPSCs) elicited by stimulating multiply-innervating CFs increase with postnatal development (Bosman et al., 2008; Hashimoto and Kano, 2003, 2005; Ohtsuki and Hirano, 2008; Scelfo and Strata, 2005). These results indicate that CF mono-innervation is established through functional strengthening of CF inputs on one hand and elimination of surplus CFs on the other hand.

In this review, we first summarize developmental process of CF synapse formation and elimination. Then, we will summarize the current understanding on molecular and cellular mechanisms for CF synapse elimination and discuss possible future directions of the research.

1. Morphological change in CF innervation during cerebellar development

Ramón y Cajal (1911) classified the development of CFs into three successive stages, i.e., the "pericellular nest or nid" stage, "capuchon" stage, and "dendritic" stage. Later, CF projection in the cerebellar cortex was investigated prior to the pericellular nest

stage (Mason and Gregory, 1984; Mason et al., 1990; Chedotal and Sotelo, 1993), and the initial stage was named the “creeper” stage by Chedotal and Sotelo (1993). We adopt P5 (the initiation of pericellular contact), P9 (the initiation of dendritic translocation), and P12 (the initiation of perisomatic CF synapse elimination and reciprocal increase of perisomatic basket cell synapses) as a hallmark of these stages in mice. These postnatal days in mice are generally consistent or retarded by a day or two as compared to studies in rats. Development of CF innervation also differs to some extents among cerebellar lobules and from PC to PC in single animals (Sugihara, 2005).

1-1. Creeper stage (P0~)

After reaching the cerebellum in the late embryonic period, CFs creep among multi-layered PC perikarya, and extend unbranched terminal arbors until reaching the border with the external granular layer (Mason et al., 1990; Chedotal and Sotelo, 1992; Morara et al, 2001). The creeper-type CFs form a few contacts on the smooth surface of immature dendritic processes. In mice at P3, CFs ascend along long dendritic processes and reach short apical dendritic processes; during the course, CFs form type 2 vesicular glutamate transporter (VGluT2)-positive terminal swellings around these dendritic processes (Fig. 1A). Each olivocerebellar axon produces about 100 creeper-type CFs, which project to a few separate terminals fields (each with 50-150 μ m wide and 400-1200 μ m long) aligned in a narrow longitudinal microzone (Sugihara, 2005).

In the creeper stage, PCs first exhibit poorly elaborated bipolar shapes and called “simple-fusiform” cells (Armengol and Sotelo, 1991). Then, as new primary dendrites grow from the apical, lateral, and basal aspects of perikarya, PCs enter the “complex-fusiform” phase. This phase peaks by P1 in rats, and gradually ends by P6.

The initial contacts between creeper-type CFs and fusiform PCs are poor in presynaptic and postsynaptic specializations, and disappear with the regression of immature dendrites (Armengol and Sotelo, 1991).

1-2. Pericellular nest stage (P5~)

In the pericellular nest stage, CFs surround the basal part of monolayered PC somata, and establish synaptic contacts with perisomatic protrusions and thorns (Sugihara, 2005) (Fig. 1B-D, 1L, 2A, 2B). Because of the extensive protrusions, PCs are in the phase of “stellate cells” (Armengol and Sotelo, 1991). Among 100 creeper-type CFs, around 10 develop into nest-type CFs (Sugihara, 2005). As each olivocerebellar axon produces, on average, 7 CFs in adulthood (Sugihara et al., 2001), CFs massively retract their terminal arbours from the creeper to the nest stage (early retraction), and the retraction further proceeds during or after the nest stage (late retraction) (Sugihara, 2005).

In rats at P5, the creeper-type, nest-type, and their transitional type of CFs are observed, representing the beginning of the pericellular nest stage (Sugihara, 2005). In mice at P5, most CF terminals are still associated with dendritic processes (arrows in Fig. 1B). However, CF terminals take up more perisomatic position than at P3; this is likely caused by the retraction of lateral and basal dendrites associated with CFs and also by the restriction of CF terminals to the basal portion of growing apical dendrites. At P5, some CF terminals form small clusters around PC somata (arrowheads in Fig. 1B), which can be taken as the initiation of pericellular nests in mice too. From P5 to P9, pericellular nests develop progressively (Fig. 1C, 1D, 2A, 2B). The density of the total perisomatic synapses peaks at P9 (Fig. 2F), when CF synapses constitute as much as

88% of the total perisomatic synapses (Hashimoto et al., 2009a; Ichikawa et al., submitted) (Fig. 2G). Thus, it is during the pericellular nest stage when CF branches display high synaptogenic activities targeting PC somata.

1-3. Capuchon stage (P9~)

The capuchon (meaning a cone-shaped ceremonial hat) stage is characterized by the displacement of CF plexus to the apical portion of PC somata. In mice, dendritic translocation of CFs starts at P9 (Fig. 1D, L) and becomes magnificent by P15 (Fig. 1F, N). Simultaneously, the major perisomatic synapses switch from CF synapse to basket cell synapse from P9 to P15 (Fig. 2G, H). During the switching period, particularly at P12 (Fig. 1E, 1M, 2C), typical feature of the capuchon stage is appreciated (Ichikawa et al., submitted). At P12, CF and basket cell synapses occupy 63% and 33% respectively of the total perisomatic synapses. It is also during the switching period that a fraction of basket cell synapses are formed on to somatic spines in addition to flat somatic surface (Fig. 2G, H) (Ichikawa et al., submitted). Furthermore, clusters of AMPA-type glutamate receptors and GABA_A receptors are clustered under single basket cell terminals (Fig. 2H). These findings suggest that, as CFs displace to apical somatic portion and dendrites, not a few somatic spines initially innervated by CFs are succeeded by basket cell fibers for a while (BF-spine synapse in Fig. 2G), during which postsynaptic receptors are reorganized from glutamatergic to GABAergic under the coverage of basket cell terminals (Fig. 2H). Thus, the capuchon stage represents dynamic transition and reorganization in the site of CF innervation site (i.e., perisomatic to peridendritic), input source of perisomatic innervation (CF to basket cell fiber), and postsynaptic receptor phenotype (glutamatergic to GABAergic).

1-4. Dendritic stage (P12~)

In the dendritic stage, CF synapses progressively translocate to growing PC dendrites (Altman, 1972; Chedotal and Sotelo, 1992; Sugihara, 2005; Hashimoto et al., 2009a) (Fig. 1E-G). In this stage, perisomatic spines contacting to CFs virtually disappear (Fig. 1J), and the fraction of CF synapses in the total perisomatic PC synapses steeply drops to 6% at P15 and <1% at P20 (Fig. 2G). Reciprocal to the loss of perisomatic CF synapses, the height of CF projection in the molecular layer increases strikingly (Fig. 1H). When the dendritic translocation is evaluated as the height of CF projection relative to the thickness of the molecular layer (37% at P9, 60% at P12, 69% at P15, and 76% at P20), it is thus clear that the translocation of CFs follows the growth of the molecular layer, most likely, the growth of PC dendrites (Fig. 1I) (Hashimoto et al., 2009a). Considering further increase of this score in adulthood (84%; Ichikawa et al., 2002), dendritic translocation of CFs can be taken as a protracted process in animal life. In this regard, it is important that dendritic translocation of CFs is an activity-dependent process, as the administration of tetrodotoxin or AMPA receptor blockers atrophies CF innervation in adult rats and mice (Bravin et al., 1999; Kakizawa et al., 2005; Cesa et al., 2007).

2. Distinct phase from multiple- to mono-CF innervation

In parallel with the developmentalons become larger among multiple CF inputs and then elimination of redundant CF synapses starts to occur. We propose four distinct phases in the process from multiple CF innervation to mono-innervation; 1) functional

differentiation among multiple CF inputs, 2) dendritic translocation of single “winner” CFs, 3) early and 4) late phases of CF synapse elimination (Fig. 3).

2-1. Functional differentiation among multiple CF inputs

Changes in relative synaptic strengths of multiple CFs innervating the same PC have been systematically studied during postnatal development by recording CF-mediated EPSCs in PCs from cerebellar slices of mice aged P2 to P21 (Hashimoto and Kano, 2003). This study shows that more than 5 discrete CF-EPSCs with similar amplitudes are recorded in PCs until around P3 (Fig. 4A, upper). In the second postnatal week, however, multiple CF-EPSCs recorded from each PC are differentiated into one large CF-EPSC and a few small CF-EPSCs (Fig. 4A, lower). These results unequivocally indicate that synaptic strengths of multiply-innervating CFs are relatively uniform in the neonatal period, and a single CF is selectively strengthened subsequently (Bosman et al., 2008; Hashimoto and Kano, 2003, 2005). Quantitative analyses of the disparity among the amplitudes of multiple CF-EPSCs in individual PCs demonstrate that one CF is selectively strengthened among multiple CFs innervating the same PC from P3 to P7 (Fig. 4B) (Hashimoto and Kano, 2003). Transient rises of glutamate concentration in the synaptic cleft are significantly higher after stimulation of the strongest CF than weaker CFs (Hashimoto and Kano, 2003). This is thought to result from the fact that the probability of multivesicular release (i.e., more than one synaptic vesicles are released simultaneously to a given postsynaptic site from the corresponding presynaptic release site) is higher for the strongest CF than for the weaker CFs.

These electrophysiological data are consistent with morphological changes in innervation pattern during this postnatal period. CFs have many creeping terminals in

the PC layer at P4, when their terminal swellings show no particular aggregation around PC somata (Sugihara, 2005). Then, from P4 to P7, CFs come to surround several specific PC somata with their terminal clusters (nest-type CFs) (Sugihara, 2005) (Fig. 1C, 2A). Pericellular nests are constructed by multiple CFs with different neuronal origins; some CFs reach target PCs as trunk fibers ascending in the granular layer, while others reach as collateral branches coming from trunk fibers that innervate neighboring PCs (Sugihara, 2005; Hashimoto et al., 2009a). Thus, pericellular nests represent the morphological basis of multiple CF innervation of PCs. Importantly, the extent of somatic coverage by respective CFs in a given pericellular nest is not even (Sugihara, 2005). To quantitatively assess the different occupancy among multiply-innervating CFs, we employed double labeling of CFs with anterograde tracer biotinylated dextran amine (BDA) injected into the inferior olive and with antibody against CF terminal marker type 2 vesicular glutamate transporter (VGluT2), and examined the composition and localization of perisomatic synapses formed by a main BDA/VGluT2-double labeled CF (yellow columns and circles in Fig. 1K-N) and other lesser CFs labeled for VGluT2 only (green columns and circles) (Hashimoto et al., 2009a). As a result, CF synapses formed by one predominant CF amount to about 60% of the total perisomatic CF synapses at P9 (Fig. 1K). Considering that individual PCs are innervated by, on the average, 3.5 CFs at P9 (Fig. 4C), each of lesser CFs is assumed to occupy <20% of the total perisomatic CF synapses. Therefore, functional differentiation among multiply-innervating CFs accompanies morphological differentiation, and both progress while they are constructing pericellular nests (Fig. 3B).

2-2. Dendritic translocation of single “winner” CFs

As described above, the site of CF innervation shifts from the soma to dendrites in postnatal development. This phenomenon is known as “CF translocation” (Altman and Bayer, 1997). We then investigated the relationship between the selective strengthening of single CFs and CF translocation (Hashimoto et al., 2009a).

The location of synapses along the somatodendritic domains of PCs can be estimated electrophysiologically by analyzing the kinetics of quantal EPSCs (qEPSCs) arising from single synaptic vesicles in CF terminals. At P7-P8 when the selective strengthening of single CFs in each PC has just completed (Fig. 4B), there is no significant difference in the distribution of rise times from 10% to 90% of peak amplitude of qEPSCs between the strongest CF and weaker CFs (Fig. 4D). Since the distribution of qEPSC rise time is affected by the somatodendritic locations of CF terminals (Roth and Häusser, 2001), synapses formed by the strongest CF and weaker CFs are thought to be located at similar locations, namely, on the soma at P7-P8 (Fig. 3A, ~P7). At P9-P10, the incidence of qEPSCs with slow rise time was more frequent for the strongest CF than for weaker CFs, suggesting the initiation of CF translocation (Fig. 3B, P9~). The difference in the distribution of qEPSC rise times becomes much larger from P11 to P14 (Fig. 4E). While the incidence of qEPSCs with slow rise time become more frequent for the strongest CF with age, the rise time of qEPSCs for weaker CFs are almost unchanged from P9 to P14 (Fig. 4D, E). These electrophysiological data collectively suggest that: (1) synaptic competition among multiple CFs occurs on the soma before P7 (Fig. 3A, ~P3 and ~P7; Fig. 3B, P3~P7), (2) only the strongest (“winner”) CF starts to translocate to dendrites at P9 and the translocation continues thereafter (Fig. 3A, ~P12; Fig. 3B, P9~), and (3) synapses of weaker (“loser”) CFs remain around the soma (Fig. 3A, ~P7 and ~P12).

These notions were tested morphologically by discriminating synapses formed by a major BDA/VGluT2-double labeled CF from those by other weaker CFs labeled for VGluT2 only (Fig. 1L-N; Hashimoto et al., 2009a). At P7, in spite of the presence of immature stem dendrite in “stellate” PCs, CFs innervate the soma only (Fig. 1C). Dendritic innervation by a main single CF starts at P9, but its innervation is still confined to the basal portion of shaft dendrites (Fig. 1D, L). At P12 and thereafter, the main CF further extends along growing PC dendrites, while leaving weaker CFs around the soma (Fig. 1E-G, M, N). These lines of electrophysiological and morphological evidence demonstrate that a single winner CF, which is selectively strengthened by P7, is only permitted to translocate to dendrites (Fig. 3A).

2-3. Early and late phases of CF synapse elimination

From P3 to P6 when functional differentiation among multiple CFs becomes larger, there is no significant reduction in the average number of CFs per PC (Fig. 4C; Hashimoto et al., 2009b). The value begins to decrease from P7 (Fig. 4C; Hashimoto et al., 2009b; Scelfo and Strata, 2005). Therefore, CF synapse elimination does not proceed in parallel with functional differentiation of multiple CFs; rather, it starts after the strengthening of single ‘winner’ CFs in individual PCs. During dendritic translocation, the strongest CF keeps its perisomatic synapses during the second postnatal week (Fig. 3A, ~P12). This is also true for weaker CFs, which remain to innervate the soma and the very basal part of dendrites (Fig. 1M). At P15, CF synapses formed by both the strongest and weaker CFs are massively eliminated from the soma, whereas dendritic innervation by the strongest CF further extends distally (Fig. 1G, N). Therefore, CF synapse elimination is achieved through non-selective pruning of

perisomatic CF synapses in the second postnatal week, and single winner CFs can thereby establish monopolized innervation in the third week (Hashimoto et al., 2009a).

Earlier studies on spontaneous cerebellar mutant mice (Crepel and Mariani 1976; Mariani et al. 1977; Crepel et al. 1980; Mariani and Changeux 1980) and animal models with experimentally-induced “hypogranular” cerebella (Woodward et al. 1974; Crepel and Delhaye-Bouchaud 1979; Bravin et al. 1995; Sugihara et al. 2000) have revealed that intact GCs and normal formation of PF-PC synapses are prerequisite for CF synapse elimination. Moreover, Crepel et al. (1981) demonstrated that the elimination of surplus CFs consists of two distinct phases, the “early phase” up to around P8 and the “late phase” from around P9 to P17 in rats (Crepel et al. 1981). In animals with “hypogranular” cerebella, the late phase of CF synapse elimination is severely impaired, while the early phase proceeds normally. This indicates that the early phase of CF synapse elimination is independent of PF-PC synapse formation, whereas the late phase critically depends on it. However, since these classical animal models have severe abnormality and deformity in the cerebellar development and structure other than GC genesis and PF-PC synapse formation, there remains a possibility that CF synapse elimination might be influenced by such developmental defects.

The analysis of mutant mice deficient in glutamate receptor $\delta 2$ (GluR $\delta 2$ or GluD2) has unequivocally revealed the PF synapse formation-independent early phase of CF synapse elimination and the PF synapse formation-dependent late phase (Hashimoto et al. 2009b). GluR $\delta 2$ is richly expressed in PCs and its deletion causes impaired PF synapse formation leading to reduced PF-PC synapse number to about half of control mice (Kashiwabuchi et al. 1995; Kurihara et al. 1997). Nevertheless, GluR $\delta 2$ deletion does not significantly affect the histoarchitecture of the cerebellum and

morphology of PCs, including dendritic branching and domains. In both GluR δ 2-knockout (KO) and control mice, the average number of CFs innervating each PC is similarly high at P5 and similarly reduced during P6-P11 (Fig. 4C). However, from P12 to P14, the average number of CFs becomes significantly larger in GluR δ 2 KO mice than in control mice (Fig. 4C, Hashimoto et al. 2009b). These results collectively indicate that CF synapse elimination in mice can be classified into the early phase from P7 to around P11 (Fig. 3B, Early phase of CF elimination, P7~P11), which is independent of PF-PC synapse formation, and the late phase from around P12 (Fig. 3B, Late phase of CF elimination, P12~P17), which requires normal PF-PC synapse formation (Hashimoto et al. 2009b). As will be described next, the early and late phases of CF synapse elimination are controlled by distinct mechanisms (Fig. 5).

3. Mechanisms for the early phase of CF synapse elimination

CF activity, elevation of intracellular Ca²⁺ concentrations in PCs, and activation of Ca²⁺-dependent intracellular signaling are considered to be the central mechanism for the early phase of CF synapse elimination (Fig. 5, top).

3-1. CF activity

Patterns of CF activity influence the early phase of CF synapse elimination. When normal activity pattern of CFs is altered by i.p. administration of harmaline to rats for 4 days from P9 to P12, multiple CF innervation persists in PCs at least until three months of age (Andjus et al., 2003). Moreover, genetic suppression of PC excitability by transgenic expression of a recombinant chloride channel during the early

phase causes multiple CF innervation that persists to at least three months of age (Lorenzetto et al., 2009). Furthermore, Bosman et al. (2008) and Otsuki and Hirano (2008) have clarified that coincident activation of PC and CF during the early phase induces long-term potentiation (LTP) exclusively at synapses with strong CF inputs. This homosynaptic LTP require a strong postsynaptic Ca^{2+} signal thought to be mediated by voltage-dependent Ca^{2+} channels (VDCCs).

3-2. P/Q-type voltage-dependent Ca^{2+} channel

Hashimoto et al. (2011) have recently reported that P/Q-type VDCCs expressed in PCs drive the early phase of CF synapse elimination. $\text{Cav}2.1$ (or $\alpha 1\text{A}$) is a pore-forming subunit of the P/Q-type VDCC, which is particularly abundant in PC dendrites, and constitutes the major Ca^{2+} current component in PCs (Mintz et al., 1992; Stea et al., 1994). In mice with PC-selective deletion of $\text{Cav}2.1$ (PC- $\text{Cav}2.1$ KO mice), the lack of $\text{Cav}2.1$ mRNA expression or P/Q-type VDCC-mediated Ca^{2+} currents is confirmed in PCs at P2 or P5, respectively. At P4-P6, no significant difference is observed in the mean number of CFs innervating each PC between PC- $\text{Cav}2.1$ KO and control mice. Thereafter, PC- $\text{Cav}2.1$ KO mice manifest severe defects in CF synapse development and elimination. First, biased strengthening of single CF inputs from P5 to P7 in control mice (Fig. 4B, G) is severely impaired in PC- $\text{Cav}2.1$ KO mice (Fig. 4H), despite comparable 4-fold increase in summed amplitudes of multiple CF-EPSCs from P5 to P8 (Fig. 4F). Second, more than one CFs translocate to dendrites in PC- $\text{Cav}2.1$ KO mice. Third, CF synapse elimination is severely impaired in PC- $\text{Cav}2.1$ KO mice until around P12. Global $\text{Cav}2.1$ KO mice replicate the aforementioned phenotypes of PC- $\text{Cav}2.1$ KO mice, i.e., normal CF innervation at P4-P6, impaired biased

strengthening of single CF inputs during the first postnatal week, and impaired subsequent elimination of surplus CFs until around P12. All these findings indicate an essential role of PC's P/Q-type VDCC in synaptic competition among multiple CFs and subsequent CF synapse elimination during the early phase (Fig. 5, top).

The physiological importance of the early phase of CF synapse elimination is appreciated well from chaotic excitatory wiring in PCs of global Cav2.1 KO mice (Miyazaki et al., 2004) and PC-Cav2.1 KO mice (Miyazaki et al., submitted) (Fig. 6, right). Innervation territory by CFs is regressed proximally to the soma and basal part of shaft dendrites. As surplus CFs innervate the proximal somatodendritic compartment (gray CF in Fig. 6, right), we refer to this form of multiple CF innervation in Cav2.1 KO mice as the “proximal type”. Innervation mode and territory by PFs also change markedly. In control mice, PF terminals are small in size, oval in shape, and form synaptic contact with single PC spines in most cases. In global Ca_v2.1 KO mice, however, a fraction of PF terminals are considerably enlarged and contact with multiple PC spines (arrowheads in Fig. 6; Miyazaki et al., 2004). These presynaptic phenotypes are reproduced in PC-Ca_v2.1 KO mice, but not in GC-Ca_v2.1 KO mice (Miyazaki et al., submitted). This finding eventually highlights that Ca_v2.1-mediated Ca²⁺ signaling and dynamics in PCs retrogradely affect the size, shape, and contact number of PF terminals. Furthermore, PF territory expands reciprocally to proximal dendrites and somata, and covers the entire somatodendritic compartment in global Ca_v2.1 KO and PC-Ca_v2.1 KO mice. As a result, characteristic features of excitatory synaptic wiring in wild-type PCs, i.e., segregated innervation territory by CFs and PFs and mono-innervation by single CFs (Fig. 6, middle), are completely disrupted in these mutant mice. Similar phenotypes are also induced after chronic blockades of neuronal activity by tetrodotoxin and AMPA

receptors by NBQX in mature cerebellum (Bravin et al., 1999; Kakizawa et al., 2005; Cesa et al., 2007). Taken altogether, strong CF activities that induce Ca^{2+} influx through P/Q-type VDCCs give competitive advantage to single winner CFs, which expels an enormous number of PF inputs to distal dendrites and eliminates surplus CFs remaining on the PC soma. This molecular function thus strengthens the functional and innervations bases for a single strongest CF, and propels the early phase of CF synapse elimination.

3-3. Insulin-like growth factor and TrkB

Insulin-like growth factor I (IGF-1) is also involved in CF synapse elimination from P8 to P12 (Kakizawa et al., 2003). IGF-1 is thought to enhance the strength of CF synapses and promote their survival, whereas the shortage of IGF-1 impairs the development of CF synapses (Kakizawa et al., 2003). In addition, Sherrard et al. (2009) reported that around the onset of the early phase of CF synapse elimination, the inferior olive and cerebellum decrease the expression of active, phosphorylated form of full-length TrkB, a receptor for brain-derived neurotrophic factor (BDNF), but increase the expression of the truncated form that acts as a negative regulator of TrkB signaling. This expression pattern is reproduced during CF degeneration following the pedunculotomy. This finding suggests that developmental decrease in TrkB signaling could be involved in the early phase of CF synapse elimination. The sites of synaptic expression and action by these growth factor and neurotrophin receptor remain largely elusive.

4. Mechanisms for the late phase of CF synapse elimination

Since normal PF-PC synapse formation is prerequisite for the late phase of CF synapse elimination, any molecules required for PF-PC synapse formation and maintenance contribute to the late phase. Among such molecules, GluR δ 2 and Cbln1 play unique roles in the formation and maintenance of PF-PC synapses, and in the construction of the structural basis for molecular mechanisms activated by PF synaptic inputs. The mGluR1-G α q-PLC β 4-PKC γ signaling cascade in PCs is driven by neural activity through the mossy fiber-to-GC/PF-to-PC pathway, and plays a central role in eliminating surplus CF synapses remaining on the PC soma (Fig. 5, bottom).

4-1 GluR δ 2

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., 2005; Kakegawa et al., 2007a, 2008). Rather, GluR δ 2 plays an important role in the formation and maintenance of the PF-PC synapse (Kashiwabuchi et al., 1995; Takeuchi et al., 2005) through its selective expression at this synapse, but not CF synapse (Takayama et al., 1995; Landsend et al., 1997), and also through specific interaction of its N-terminal domain with presynaptic neurexin via Cbln1 (Hirai et al., 2005; Matsuda et al., 2010; Uemura et al., 2010). Primary defects in GluR δ 2 KO mice or spontaneous GluR δ 2 mutant *hotfoot* mice occur in the connectivity of PF-PC synapses, as manifested by the emergence of free spines and the mismatching of pre- and postsynaptic specializations (Guastavino et al., 1990; Kurihara et al., 1997; Lalouette et al., 2001). Free spines are numerous in the distal dendritic domain innervated by PFs only (56% of the total spines; green region in Fig. 6, left) and also found in the intermediate domain receiving mixed PF and CF innervation (37%; light

blue), whereas no free spines are found in the proximal domain innervated by CFs only (purple) (Ichikawa et al., 2002). This indicates that free spines are generated by failed synaptic contact with PF terminals. In PCs of wild-type mice, the density of synaptic AMPA receptors is approximately 5 times lower at PF synapse than at CF synapse, but this biased synaptic expression is disrupted by 3-to-5-fold increase of synaptic AMPA receptors at PF synapse in GluR δ 2 KO mice (Yamasaki et al., 2011). Therefore, GluR δ 2 potently strengthens structural connectivity of PF-PC synapses, while it weakens functional strength of the same synapses by reducing postsynaptic AMPA receptor number.

Defects in PF-PC synapse formation and stabilization also severely alter CF innervation in GluR δ 2 KO mice. Distal extension of CF territory is demonstrated by increased CF distribution in the molecular layer of GluR δ 2 KO mice (up to 95% of the molecular layer) compared to control mice (84%) (Ichikawa et al., 2002). This is caused by extension of CFs along and beyond dendritic trees of a given PCs to take over free spines on distal dendrites. Such an aberrant extension eventually produces a number of ectopic CF synapses targeting distal dendrites, and causes a “distal type” of multiple CF innervation (gray CF in Fig. 6, left). This morphological observation is consistent with Ca²⁺ imaging combined with electrophysiological recording. In GluR δ 2 KO mice, a single strong CF elicits large EPSCs with fast rise time and high Ca²⁺ elevation spreading throughout the dendritic tree, whereas weak CFs elicit small EPSCs with slow rise time and low Ca²⁺ elevation confined to local distal dendrites (Hashimoto et al., 2001). These unique phenotypes in GluR δ 2 KO mice indicate that PFs compete for the innervation territory with CFs during development. In this heterosynaptic type of competition, GluR δ 2 selectively strengthens PF synapses at distal dendrites, and

restricts CF innervation to proximal dendrites. This heterosynaptic mechanism for CF territory restriction plays an essential role in the establishment of CF mono-innervation in the late phase by rather non-selective elimination of CF synapses remaining on the PC soma.

According to a recent report by Kaneko et al. (2011), the arborization of PC dendrites undergoes dynamic remodeling from multiplaner to monoplaner configuration during the third and fourth postnatal weeks in mice (Kaneko et al., 2011). They also report that distal portions of minor dendritic arbors of multiplanar PCs are often associated with collaterals of main CF branches, suggesting that distal type of CF innervation occurs frequently during normal cerebellar development. However, small CF-EPSCs with slow rise times, which reflect the distal type of CF innervation, are rarely recorded in wild-type PCs (Hashimoto et al., 2001). Therefore, it is uncertain whether the CF collaterals can form functional synapses on minor dendritic arbors of multiplanar PCs in normal development.

4-2 Cbln1

Cbln1 was originally identified as a precursor of PC-specific peptide cerebellin, thus being originally termed precerebellin (Slemmon et al., 1984). However, C-terminal two-thirds of Cbln1 share significant structural similarity to the globular domain of complement C1q chain (Urade et al., 1991; Kishore & Reid, 2000). Cbln1 is highly expressed in cerebellar GCs, released to the extracellular space, and highly accumulated in the synaptic cleft of PF-PC synapses (Bao et al., 2005; Hirai et al., 2005; Miura et al., 2009).

Cooperative role of Cbln1 and GluRδ2 was first indicated by similar cerebellar

phenotypes between Cbln1 KO and GluR δ 2 KO mice, including free spines, mismatching of pre- and postsynaptic specialization at PF synapses, distal extension of CF territory, severe persistence of multiple CF innervation at distal dendrites, and impaired long-term depression (Hirai et al., 2005) (Fig. 6, left). It is now widely known that Cbln1 is a unique bidirectional synaptic organizer. Cbln1 directly binds to the N-terminal domain of GluR δ 2 and promotes clustering of postsynaptic molecules, while it also binds to presynaptic neurexins carrying the splice site 4 insert and induces presynaptic differentiation (Matsuda et al., 2010; Uemura et al., 2010; Matsuda and Yuzaki, 2011). Therefore, trans-synaptic interaction of postsynaptic GluR δ 2 and presynaptic neurexins via Cbln1 mediates synaptic connectivity between PF terminals and PC spines. This molecular function provides a structural basis for mGluR1-G α q-PLC β 4-PKC γ signaling in PCs that is driven by PF synaptic activity and plays a central role for eliminating redundant CF synapses on the PC soma (see below). Importantly, the GluR δ 2-Cbln1-neurexin system is also essential to maintain the PC circuitry in adulthood, as the characteristic phenotypes in GluR δ 2 KO or Cbln1 KO mice are restored by rescue experiments (Ito-Ishida et al., 2008; Torashima et al., 2009), and are reproduced by molecular ablation in adulthood (Takeuchi et al., 2005; Miyazaki et al., 2010).

4-3 mGluR1 signaling pathway

During normal development, PCs richly express mGluR1 of the group I mGluR, G α q of the heterotrimeric Gq family, type 3 and 4 isoforms of the phospholipase C β (PLC β 3 and PLC β 4), and the γ subtype of the protein kinase C (PKC γ) (Saito et al., 1988; Shigemoto et al., 1992; Tanaka et al., 2000; Watanabe et al., 1998; Nakamura et

al., 2004; Nomura et al., 2006). Multiple CF innervation persists into adulthood in null-mutant mice deficient in mGluR1, G_{aq}, PLC β 4, or PKC γ , whereas dendritic trees of PCs and PF synapse formation are normal in all these mice (Hashimoto et al., 2000; Ichise et al., 2000; Kano et al., 1995; Kano et al., 1997; Kano et al., 1998; Offermanns et al., 1997; Levenes et al., 1997). Electrophysiological examination demonstrates that the elimination of surplus CF synapses occurs normally during the first and early second postnatal weeks in the four mouse strains. However, these mice display abnormal synapse elimination toward the end of the second postnatal week. These results suggest that the signaling cascade from mGluR1 to PKC γ is essential for the late phase of CF synapse elimination, but dispensable for the early phase of CF synapse elimination (Fig. 5, bottom). Importantly, the formation and function of PF-PC synapses are normal in these mutant mice. Therefore, impaired CF synapse elimination is not caused secondarily by the defect in PF synaptogenesis.

The defect of CF synapse elimination in mGluR1 KO mice is restored by selective transgenic expression of mGluR1a in PCs (Ichise et al., 2000). PC-specific expression of a PKC inhibitor peptide also impairs regression of CF synapses (De Zeeuw et al., 1998). Furthermore, intracerebellar localization of multiply-innervated PCs in PLC β 4 KO mice exactly matches with that of PCs predominantly expressing PLC β 4 in control mice (Kano et al., 1998). These lines of evidence further support that the mGluR1-to-PKC γ signaling cascade in PCs, but not other cell types, plays a central role in the late phase of CF synapse elimination.

The mGluR1 signals required for the late phase of synapse elimination are thought to be driven by PF activity, since mGluR1 can readily be activated by PF inputs (Batchelor et al., 1994; Finch and Augustine, 1998; Takechi et al., 1998). Furthermore,

chronic blockade of cerebellar NMDA receptors results in the impairment of CF synapse elimination (Rabacchi et al., 1992) specifically in its later phase (Kakizawa et al., 2000). NMDA receptors are not expressed at either PF or CF synapses in PCs at this age, but are abundantly expressed at mossy fiber-GC synapses (Kakizawa et al., 2000; Yamada et al., 2001). Therefore, chronic blockade of cerebellar NMDA receptors should affect mossy fiber to GC transmission. Although NMDA receptors are reported to be present in PCs during early development and in adulthood (Renzi et al., 2007; Piochon et al., 2007), they do not seem to contribute to CF synapse elimination. Taken together, neural activity along the mossy fiber-to-GC /PF-to-PC pathway and the subsequent activation of the mGluR1 signaling cascade in PCs are the key mechanism for the late phase of CF synapse elimination (Fig. 5, bottom) (Kakizawa et al., 2000).

4-4 Other molecules

Genetic or pharmacological deletion in mice of TrkB (Bosman et al., 2006; Johnson et al., 2007), myosin Va (Takagishi et al., 2007), glutamate transporter GLAST (Watase et al., 1998), or novel brain-specific receptor-like protein family BSRP (Miyazaki et al., 2006) also impairs CF synapse elimination in the second postnatal week. Numerous free spines as well as distal extension of CF territory is also reported in spontaneous ataxic mutant rigoletto (*rig*) (also known as waddles; *wdl*) (Hirasawa et al., 2007), which is caused by a 19 bp deletion in exon 8 of carbonic anhydrase *Car8* gene (Jiao et al., 2005). However, it has not been examined whether CF synapse elimination is normal or not.

Null mutant mice deficient in Ca^{2+} /calmodulin-dependent protein kinase IV (CaMKIV) are reported to have persistent multiple CF innervations, but it is unclear at which stage of postnatal development the impairment occurs (Ribar et al., 2000). Null

mutant mice deficient in α -calcium/calmodulin-dependent protein kinase II (α CaMKII) also display multiple CF innervations at P21-P28 (Hansel et al., 2006). Since this phenotype disappears in adulthood, α CaMKII deficiency delays, but not prevents, CF synapse elimination.

5. Homosynaptic and heterosynaptic competitions

The elucidation of molecular mechanisms involving the early and late phases of CF synapse elimination has substantiated that these mechanisms work in concert to promote homosynaptic competition among multiple CFs and heterosynaptic competition between PF and CF (Fig. 5). In early postnatal days, CFs from multiple neuronal origins converge on the soma of the same PCs, and their neural activities inducing P/Q-VDCC-mediated Ca^{2+} influx are the central mechanism for the early phase of CF synapse elimination. In the first postnatal week, PC-Ca_v2.1 KO mice are severely impaired in biased strengthening of single CF inputs (Fig. 4H), whereas they are normal in the mean number of CFs innervating each PC and in the summed amplitudes of multiple CF-EPSCs (Fig. 4F). Obviously, these results indicate that the enhancement of the total CF synaptic strengths and the biasing of individual synaptic strengths towards single selected inputs are based on different mechanisms. Although molecular bases for the mechanisms are currently unknown, it can be assumed that developmental change in the amount of *resource* necessary for maintaining synaptic efficacy and developmental competition for such *resource* by multiple inputs are two important factors (Sanes and Lichtmann, 1999; Goda and Davis, 2003). As the amount of *resource* increases with postnatal development, the total CF synaptic efficacy, as reflected in summed amplitudes of multiple CF-EPSCs (Fig. 4F), becomes larger. This process itself is

independent of P/Q-type VDCCs, but activity-dependent assignment of the *resource* to respective CFs depends on P/Q-type VDCCs. Stronger CFs can activate postsynaptic P/Q-type VDCCs more effectively and may gain more *resource* than weaker CFs. This homosynaptic competition results in selective strengthening of a single “winner” CF that can translocate to dendrites, and in the weakening of the rest of CFs that remain in perisomatic sites. The promotion of dendritic translocation by the single “winner” CF will then fuel heterosynaptic competition with PF inputs for innervation territory on dendritic trees of PCs.

In the late phase of CF synapse elimination, PC dendrites grow dynamically and an enormous number of PF synapses come into existence (Woodward et al., 1971; Altman, 1972). Trans-synaptic interaction of the GluR δ 2-Cbln1-neurexin system promotes the formation and maintenance of PF synapses on PC dendrites. The significance of this molecular function is two-fold (Fig. 5). First, complete occupancy of PC spines by PF synapses in distal dendrites eventually restricts CF innervation to proximal dendrites. This will provide a structural basis that fuels heterosynaptic competition with CF inputs for the innervation territory, which might in turn reduce the total amount of *resource* given to CF synapses. Second, owing to the establishment of PF-PC synapse formation, PF activity involving NMDA receptors at mossy fiber-GC synapses can activate the mGluR1-to-PKC γ signaling cascade in PCs enough to produce certain *signal* promoting CF synapse elimination. Although molecular bases for the mGluR1-driven *signal* are currently unknown, the *signal* will provide a functional basis for non-selective elimination of perisomatic CF synapses in the late phase. Through the homo- and heterosynaptic competitions, PCs can establish both segregated innervation territory by CFs and PFs and CF mono-innervation.

6. Future directions

The scheme in Fig. 5 further highlights important questions to be answered in future studies. How does the P/Q-type VDCC differentially assign the *resource* among CF synapses in an activity-dependent manner? How can the *resource* strengthen the strongest CF input, while it weakens lesser inputs? Likewise, how can mGluR1-driven *signal* conduct non-selective pruning of perisomatic CF synapses, while it spares dendritic CF synapses? Why are the dual mechanisms, i.e., the P/Q-type VDCC and mGluR1 signaling cascade, necessary to accomplish the developmental elimination of perisomatic CF synapses? What is the final event that executes the pruning of waning CF synapses? Above all, molecular identification of such *resource* and *signal* will be the most important issue to be clarified in future studies. Before closing this review, we will depict some of relevant findings that might help us solve these questions in future.

Chronic blockades of neuronal activities and AMPA receptors not only shift heterosynaptic competition to the disadvantage of single main CFs (Bravin et al., 1999; Kakizawa et al., 2005; Cesa et al., 2007), but also reduce glutamate concentration transient in the synaptic cleft and also the frequency of quantal EPSCs at CF-PC synapses (Kakizawa et al., 2005). This suggests that some retrograde signaling mechanisms exist to inform respective presynaptic terminals of the state of postsynaptic activities, and further weaken the synaptic basis of lesser inputs. CF activity leading to P/Q-type VDCC-mediated Ca^{2+} influx might link to the production of such retrograde signals. In young adult cerebellum, chronic blockade of mGluR1 or inositol 1,4,5-trisphosphate (IP_3) signaling in PCs is reported to decrease glutamate release probability from PFs (Furutani et al., 2006). BDNF is suggested to mediate the

retrograde signaling for the maintenance of PF function (Furutani et al., 2006). Therefore, it would be interesting to test whether neurotrophins and cytokines mediate retrograde signaling for activity-dependent strengthening and maintenance of CF-PC synapses.

A steep decrease of perisomatic CF synapses occurs from P9 to P15 (Fig. 1J). The decrease accompanies reciprocal increase of basket cell synapses and the switching of receptor phenotypes in PC somata (Ichikawa et al., submitted) (Fig. 2G). The development of the powerful perisomatic inhibition could be related to differential actions of the mGluR1-driven *signal* between perisomatic and dendritic CF synapses. In this regard, the involvement of TrkB in the late phase of CF synapse elimination is interesting, because TrkB signaling is required for normal development of GABAergic innervation and transmission in PCs. In TrkB KO mice, the number of GABAergic synapses is reduced and the inhibitory postsynaptic currents are prolonged presumably due to impaired $\alpha 3$ -to- $\alpha 1$ subunit switching (Bosman et al., 2006). It seems therefore important to test the role of perisomatic GABAergic synapse formation in non-selective pruning of perisomatic CF synapses.

It has been reported that waning CF synapses on PC somata and shaft dendrites are surrounded by ring-like structures with elevated lysosomal activity (Song et al., 2008). The structures are assumed to be Bergmann glia, and emerge abundantly in the cerebellar cortex during the second and third postnatal weeks. During synapse elimination of neuromuscular junction, similar structures in Schwann cells engulf bulb-shaped tips of retreating motor axons and their fragments “axosomes” (Bishop et al. 2004; Song et al. 2008). Therefore, it is possible that waning CF synapses might be tagged and recognized by nearby glia for final digestion in a manner similar to the

retreating motor axons at neuromuscular junction. Interesting with this point, somatic spines initially innervated by CFs are not only taken over by basket cell fibers, but also surrounded by Bergmann glia (Ichikawa et al., submitted).

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Figure Legends

Fig. 1. Postnatal development showing CF translocation to growing PC dendrites in C57BL/6 mice. *A, B.* Double immunofluorescence for VGluT2 (CF terminal marker, red) and calbindin (PC marker, green) at P3 (A) and P5 (B). At these stages, VGluT2-labeled CF terminals are mainly associated with calbindin-labeled PC processes (arrows). At P5, CF terminals take up more perisomatic position than at P3, and some terminals start to form clusters (arrowheads) around PC somata (asterisks).

C-G. Fluorescent labeling for anterograde tracer biotinylated dextran amine (BDA, red) and calbindin (green) at P7 (C), P9 (D), P12 (E), P15 (F), and P20 (G). Pericellular nests are developed at P7 and P9 (pericellular nest stage). Then, perisomatic CF innervation is shifted to the apical side of PC somata at P12 (capuchon stage), and mostly to dendrites at P15 and P20 (dendritic stage). Note progressive dendritic translocation from P9 to P20. *H, I.* Developmental changes in the mean CF reach (H) and its relative height in the molecular layer (I). Values are mean \pm S.D. for 10 to 12 CFs labeled with BDA from 3 mice at each age. CF reach was measured from the apical pole of PC somata to the tips of tracer-labeled CFs. *J.* The density of somatic spines forming CF synapses per 1 μm of CF height. Values are mean \pm S.D. *K.* The composition of perisomatic CF synapses innervated by a single main BDA-labeled/VGluT2-labeled CF (yellow) and other BDA-unlabeled/VGluT2-labeled CFs (green). *L-N.* CF wiring reconstructed from serial electron microscopy at P9 (L), P12 (M), and P15 (N). Yellow lines and circles on the right side of each PC represent BDA-labeled/VGluT2-labeled single main CF, while green circles on the left side represent BDA-unlabeled/VGluT2-labeled weaker CFs. At P9 and P12, a single main CF translocates to dendrites, while keeping its perisomatic synapses together with those

by weaker CFs. Most of perisomatic CF synapses are eliminated at P15. Figure 1A and 1B are kindly provided by Dr. R. Ichikawa in Sapporo Medical University School of Medicine. Figure 1C-N is modified from Hashimoto et al, Neuron, 63:106-118, 2009. Scale bars, 20 μ m.

Fig. 2. Developmental switching in perisomatic PC innervation from CFs to basket cell fibers in C57BL/6 mice. *A-E*. Triple fluorescent labeling for inhibitory terminals with vesicular inhibitory amino acid transporter (VIAAT) antibody (green, *A1-E1*), CFs with anterograde tracer BDA (red, *A2-E2*), and PCs with calbindin antibody (blue, both panels). Asterisks indicate PC somata. Double and single arrowheads indicate the tip or trajectory of CF projection, respectively. Large arrowheads in *E* indicate the pinceau formation surrounding the axon initial segment of PCs. *F*. The density of the total perisomatic synapses. The density is expressed as the number per 100 μ m² of somatic surface area. *G*. The composition of four types of perisomatic synapses; basket fiber-PC soma synapse (BF-soma, light green), basket fiber-PC spine synapse (BF-spine, green), and CF-PC spine synapse (CF-spine, red), and other somatic synapse (blue). *H*. Summary illustrations on developmental switching of perisomatic innervation and receptor phenotype in PCs. At P7 and P9, the predominance of CF-spine (CF-Sp) synapses corresponds to the pericellular nest stage of CF innervation. At P12, perisomatic CF-spine synapses are displaced to the apical somatic portion and shaft dendrites (the capuchon stage), whereas BF-spine synapses (BF-Sp) and BF-soma synapses (BF-Sm) increase reciprocally. At this stage, fragmental GluA2 and GABA_{AR} α 1 clusters co-exist under the same BF terminals, particularly, at basal somatic portion. At P15, the major perisomatic synapses are BF synapses composed of

BF-spine and BF-soma synapses, and most CF-spine synapses are eliminated from PC somata (the dendritic stage). At P20, most perisomatic synapses become BF-soma synapses and the pinceau is established, thus attaining the adult type of innervation. Free spines surrounded by BFs (FS-BF) or BG (FS-BG) also appear transiently during the period of CF-to-BF switchover. Modified from Ichikawa et al. (in submission). Scale bars, 20 μ m.

Fig. 3. Four developmental phases in the process from multiple innervation to mono-innervation of PCs by CFs. Until P3, synaptic strengths of multiply-innervating CFs are relatively uniform. From P3 to P7, one CF is selectively strengthened, thus being termed the phase of “functional differentiation.” From P9 on, the strongest CF undergoes dendritic translocation (the phase of “CF translocation”). On the other hand, the average number of CFs per PC shows no significant reduction from P3 to P6 (>5 CFs), and progressively decreases from P6. Electrophysiological analysis on GluR δ 2 KO mice demonstrates the “early phase of CF synapse elimination” during P7-P11, which is independent of PF-PC synapse formation, and the “late phase of CF synapse elimination” during P12-P17, which requires normal PF-PC synapse formation.

Fig. 4. Electrophysiological evidence for functional differentiation, synapse elimination and translocation of CFs. *A.* CF-EPSCs recorded in a PC at P3 (upper) or at P12 (lower). Two to three traces are superimposed at each threshold stimulus intensity. Holding voltage (V_h) was -80 mV for P3 and -20 mV for P12. *B.* Developmental changes in the Disparity Ratio. The amplitudes of individual CF-EPSCs in a given multiply-innervated PC were measured at the same V_h and they were numbered in the order of their

amplitudes ($A_1, A_2, \dots, A_i, \dots, A_N$, $N \geq 2$, N is the number of CFs innervating a given PC.

A_i is the EPSC amplitude for the CF_i. A_N represents the EPSC amplitude for the strongest CF). Disparity Ratio was calculated by the following formula;

$$\text{Disparity Ratio} = \frac{\sum_{i=1}^{N-1} \frac{A_i}{A_N}}{N-1} \quad (N \geq 2)$$

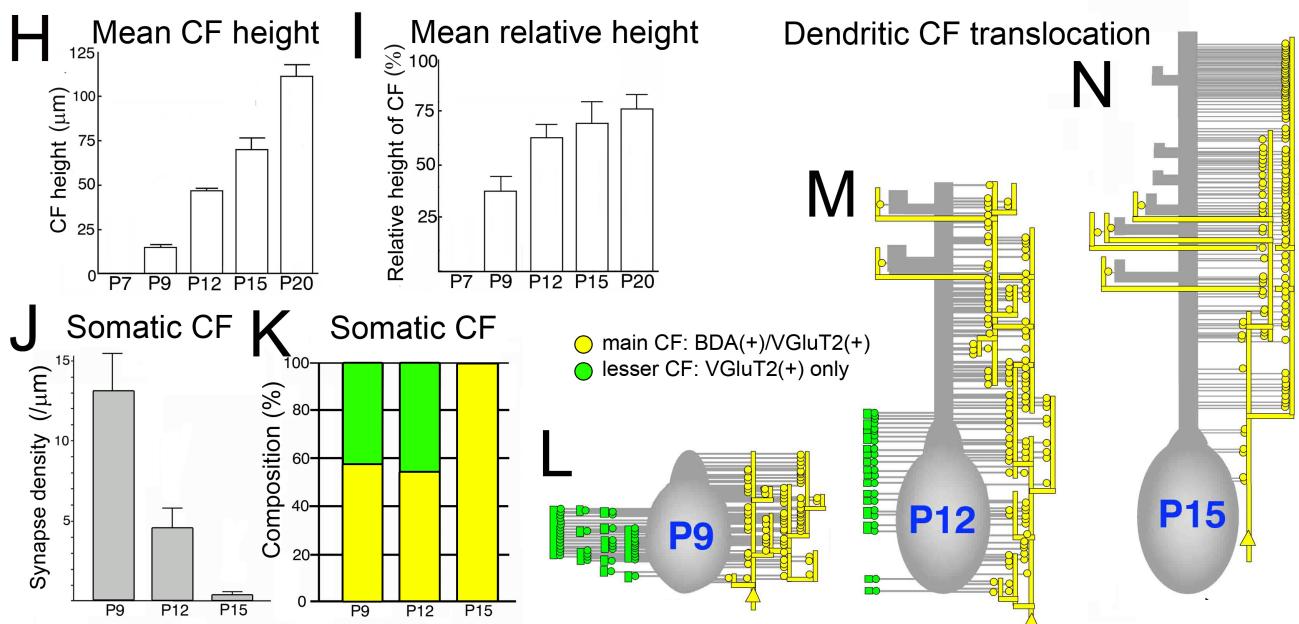
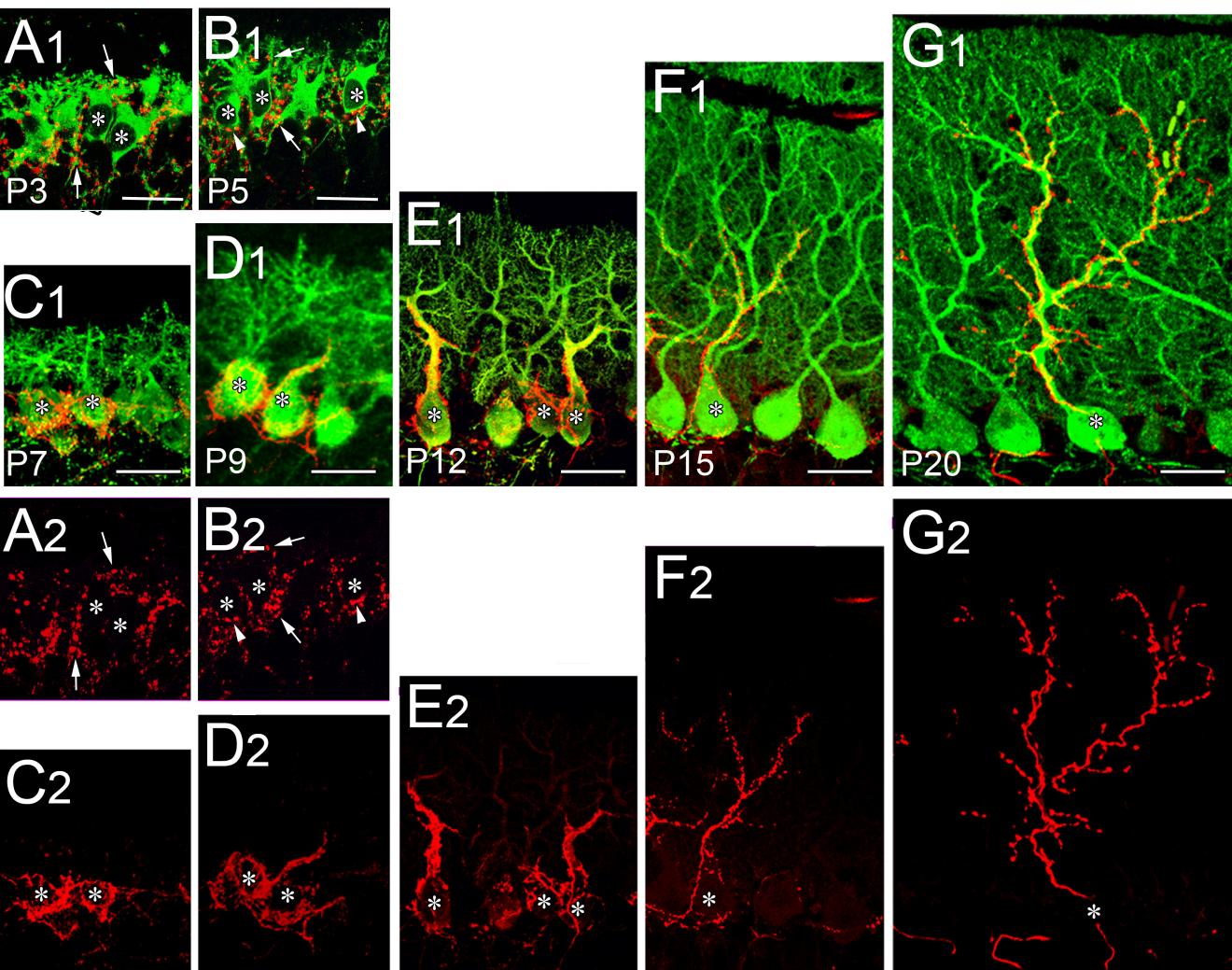
Data for P9–P11 and those for P12–P14 are pooled and indicated with filled triangles. Data for P15 to P21 are pooled and indicated with filled boxes. *C*. Postnatal changes in the averaged number of CFs innervating each PC in C57BL/6 mice (filled blue), control mice for GluD2 KO mice (open black) and GluD2 KO mice (filled black). The values of GluD2 knockout mice are significantly larger than control at P12, P13 and P14 (**, p<0.01, Mann-Whitney U-test). *D, E*. Average cumulative histograms for the 10–90% rise times of qEPSCs arising from CF-mono (green), CF-multi-S (pink) and CF-multi-W (blue) at P7-P8 (*D*) and P11–P14 (*E*). *F*. Postnatal changes in the total amplitudes of CF-EPSCs (at $V_h = -20$ mV) elicited in each PC in control (open) and PC-Cav2.1 KO (gray) mice. *G, H*. Postnatal changes in the fractions of the largest (blue), second (pink), third (green), and fourth (violet) EPSC amplitudes relative to the total CF-EPSC amplitude in control (*G*) and PC-Cav2.1 KO (*H*) PCs. Values are expressed as mean \pm sem. *A* and *B* are modified from Hashimoto and Kano, Neuron, 38, 785-796, 2003. *C* is modified from Hashimoto et al., Neuroscience, 162, 601-611.2009b. *D* and *E* are modified from Hashimoto et al., Neuron, 63:106-118, 2009a. *F, G* and *H* are modified from Hashimoto et al. Proc. Natl. Acad. Sci. USA. 108, 9987-9992, 2011.

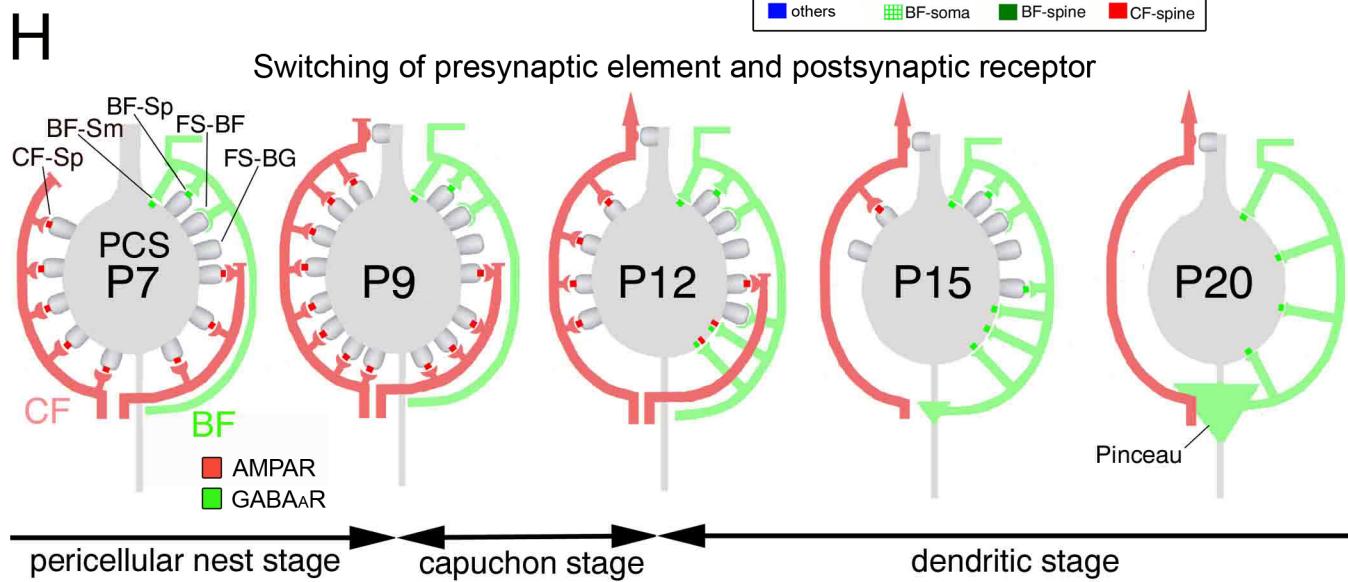
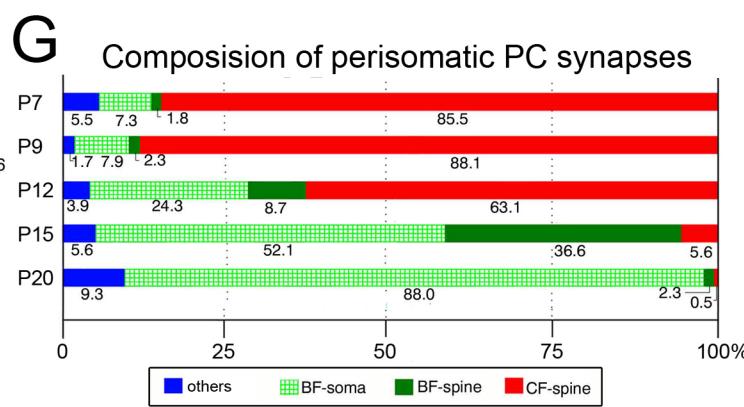
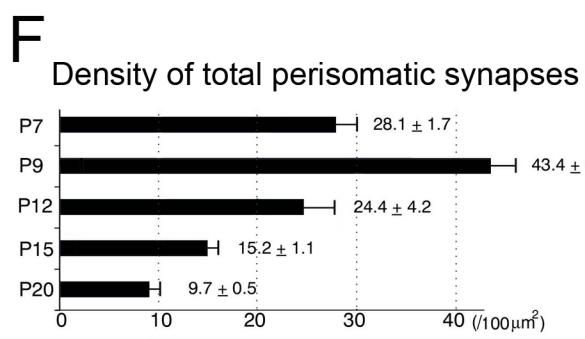
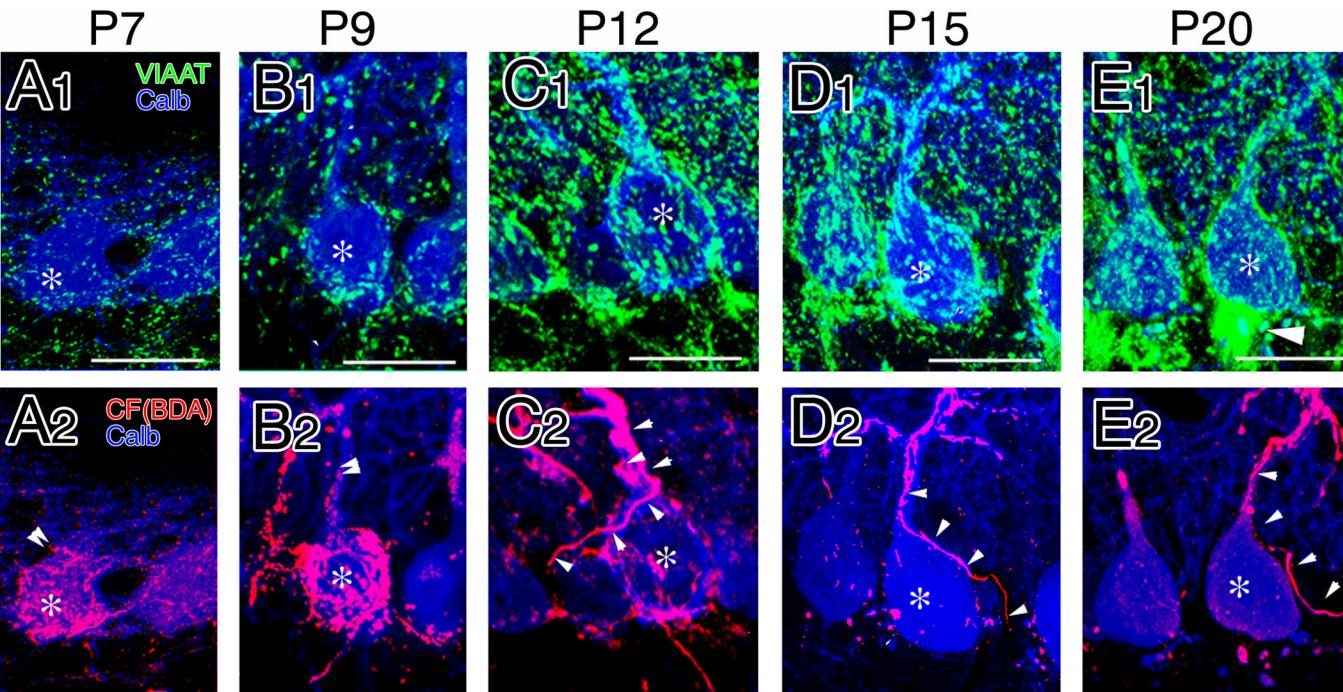
Fig. 5. Cellular and molecular mechanisms underlying the early and late phase of CF synapse elimination in early postnatal development. *Top*, Early phase of CF synapse

elimination. CF activities leading to Ca^{2+} influx through the P/Q-type VDCC will assign the *resource* (?) to respective CF synapses in an activity-dependent manner. This promotes functional differentiation and fuels homosynaptic competition among multiply-innervating CF inputs. As a result, the strongest CF is only permitted to translocate to dendrites, which then fuels heterosynaptic competition with PF inputs. Other weaker CFs remain on the soma, until their perisomatic synapses are massively eliminated. *Bottom*, Late phase of CF synapse elimination. In the late phase of CF synapse elimination, PF-PC synapses play two important roles with each being conducted by different molecular mechanisms. First, trans-synaptic interaction of presynaptic neurexin and postsynaptic GluR δ 2 via Cbln1 consolidate structural connectivity of PF-PC synapses at distal dendrites, which eventually restrict CF innervation to proximal dendrites. Second, neural activities transmitted along the mossy fiber-to-GC/PF-to-PC pathway activate the mGluR1-to-PKC γ signaling cascade in PCs. In the downstream of this cascade, certain *signals* (?) are produced to drive non-selective elimination of perisomatic CF synapses. TrkB, myosin Va, BSRP and GLAST are also involved in the late phase of CF synapse elimination. Modified from Kano et al, Phil. Trans. R. Soc. B 363, 2173-2186, 2008.

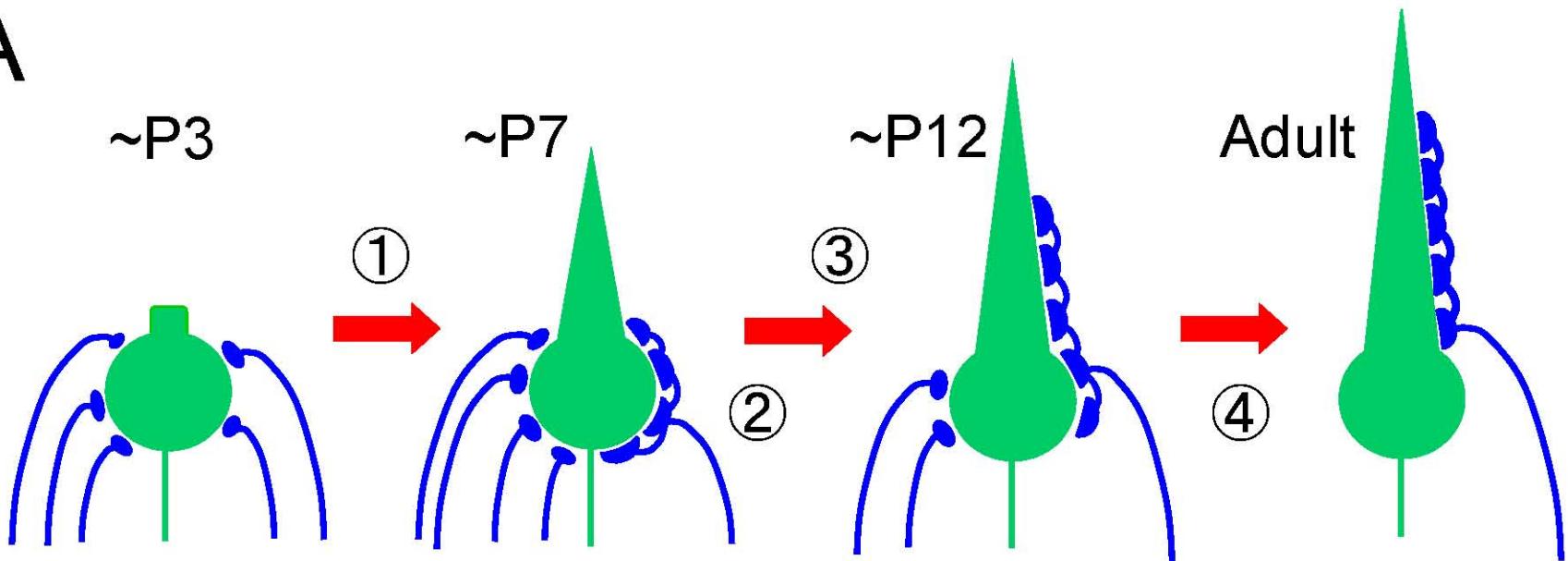
Fig. 6. Summary diagram illustrating molecular mechanisms for competitive synaptic wiring in PCs. In wild-type animals, a single main CF (black beaded fiber) monopolizes the proximal domain of PC dendrites (purple) and establishes hundreds of glutamatergic synapses, while numerous PFs innervate the distal dendritic domain (green) and form 1:1, or 1:2, contact ratio with PC spines (middle). Note opposite shift of CF and PF territories between GluR δ 2/Cbln1 KO (left) and Cav2.1 KO (right) mice. When the

territories are shifted to either direction, innervation of surplus CFs (gray beaded fibers) is permitted to cause multiple CF innervation at distinct dendritic domains, and the intermediate domain of PC dendrites (light blue), which innervated by both CFs and PFs, expands. Furthermore, in Cav2.1 KO, a fraction of PF terminals are enlarged and make contact with several PC spines (arrowheads). Although synaptic wiring in mGluR1 KO mice is not illustrated in this diagram, the mGluR1 signaling cascade mediates PF synapse activities to lead to the pruning of surplus CFs. Armed with all these mechanisms, CF and PF territories are sharply bordered and aberrant innervation by surplus CFs is prevented in wild-type animals (middle). Modified from Watanabe, Tohoku Journal of Experimental Medicine 214:175-190, 2008.

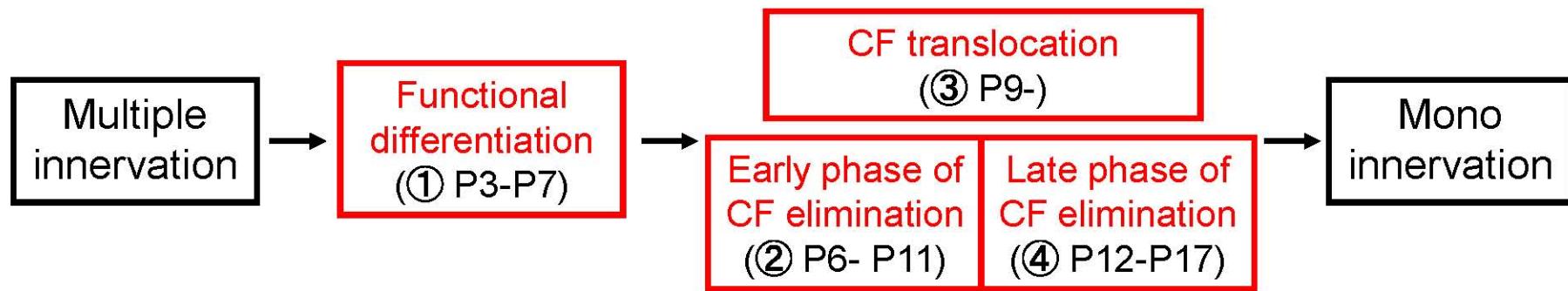


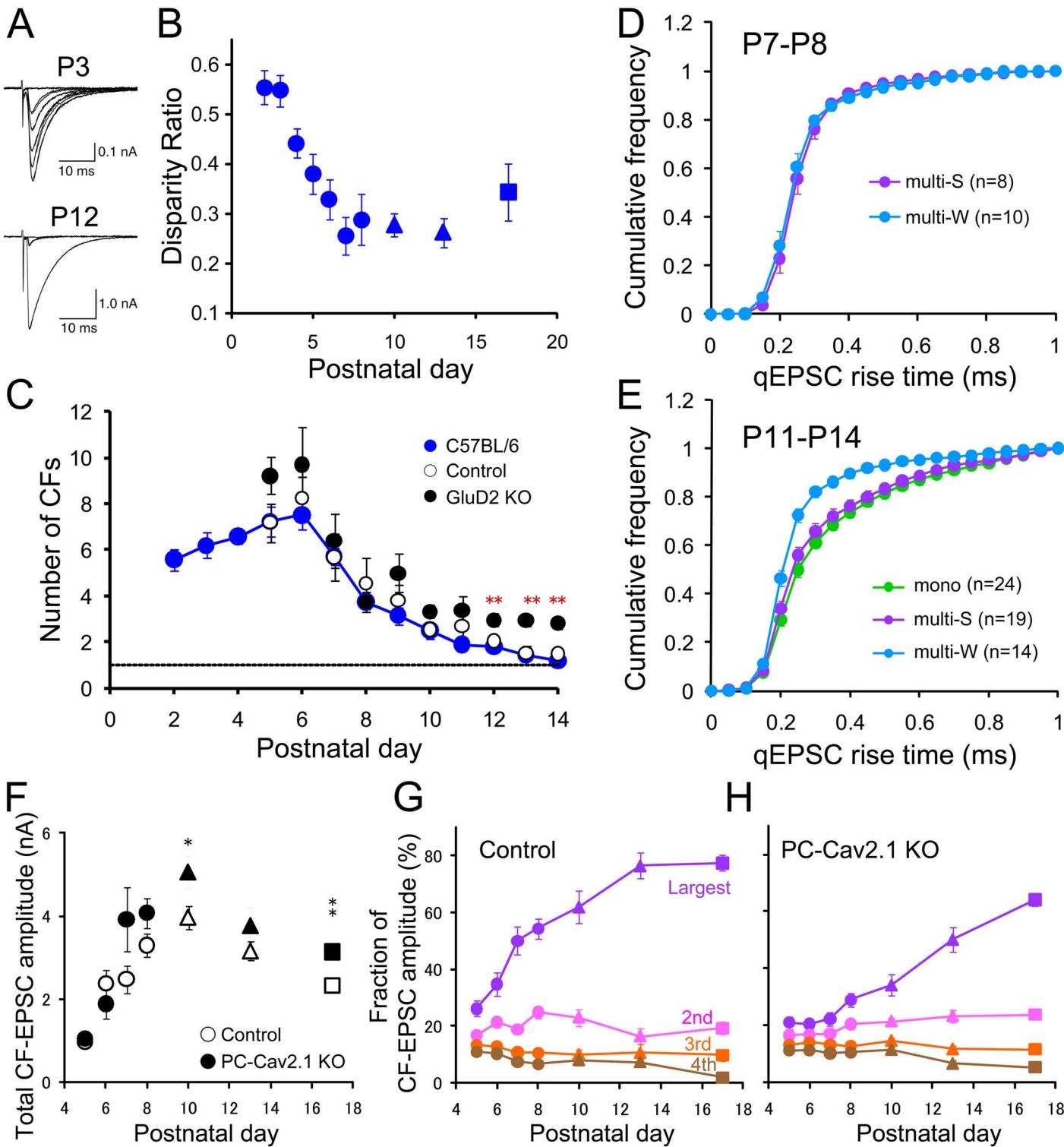


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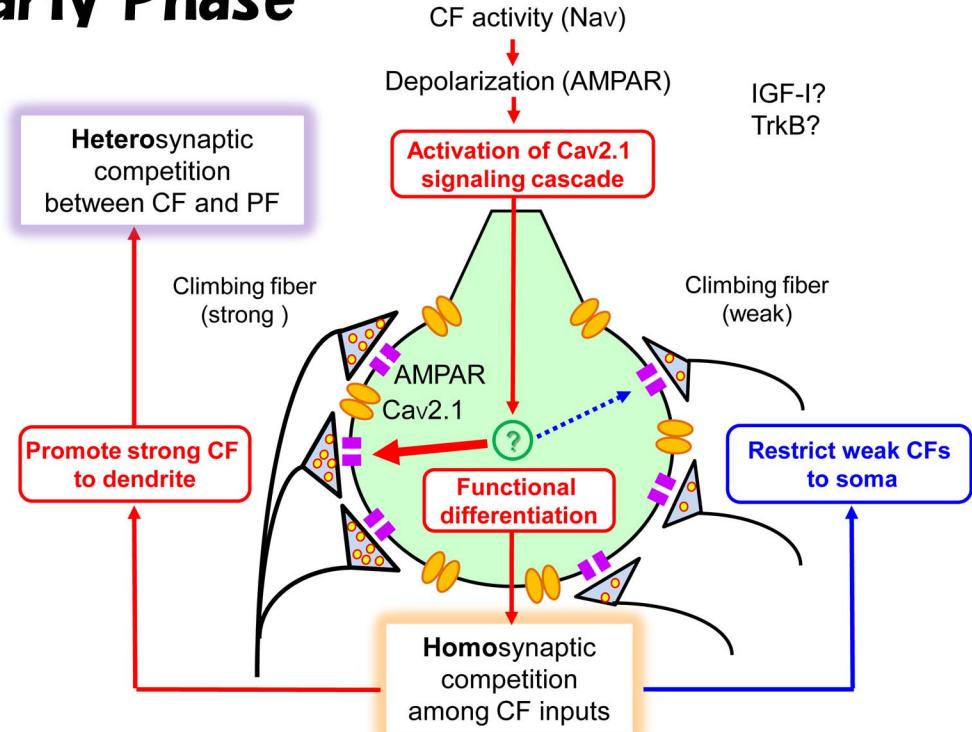


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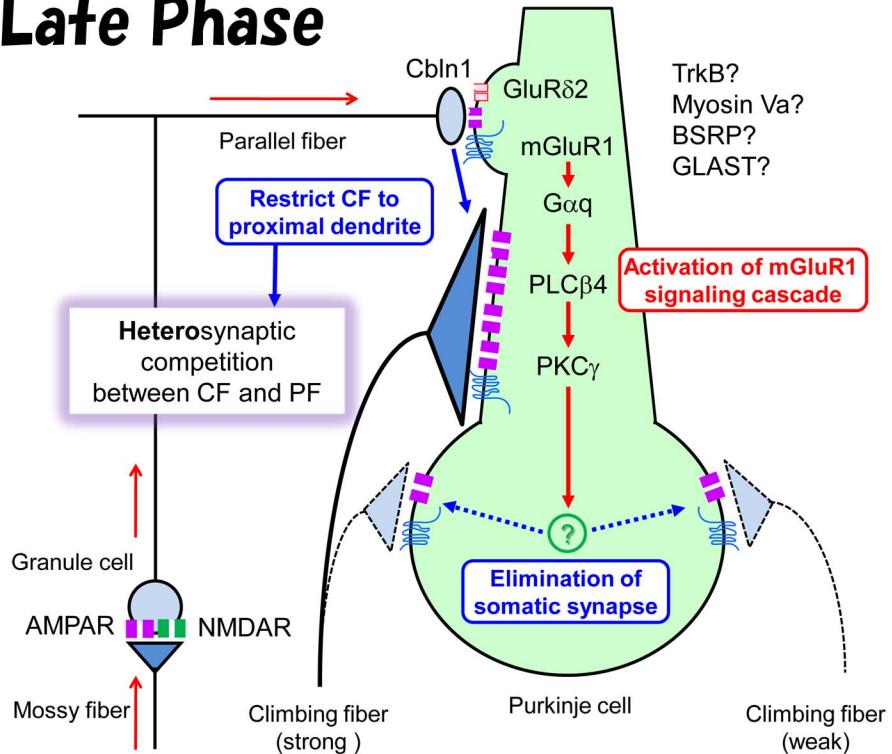


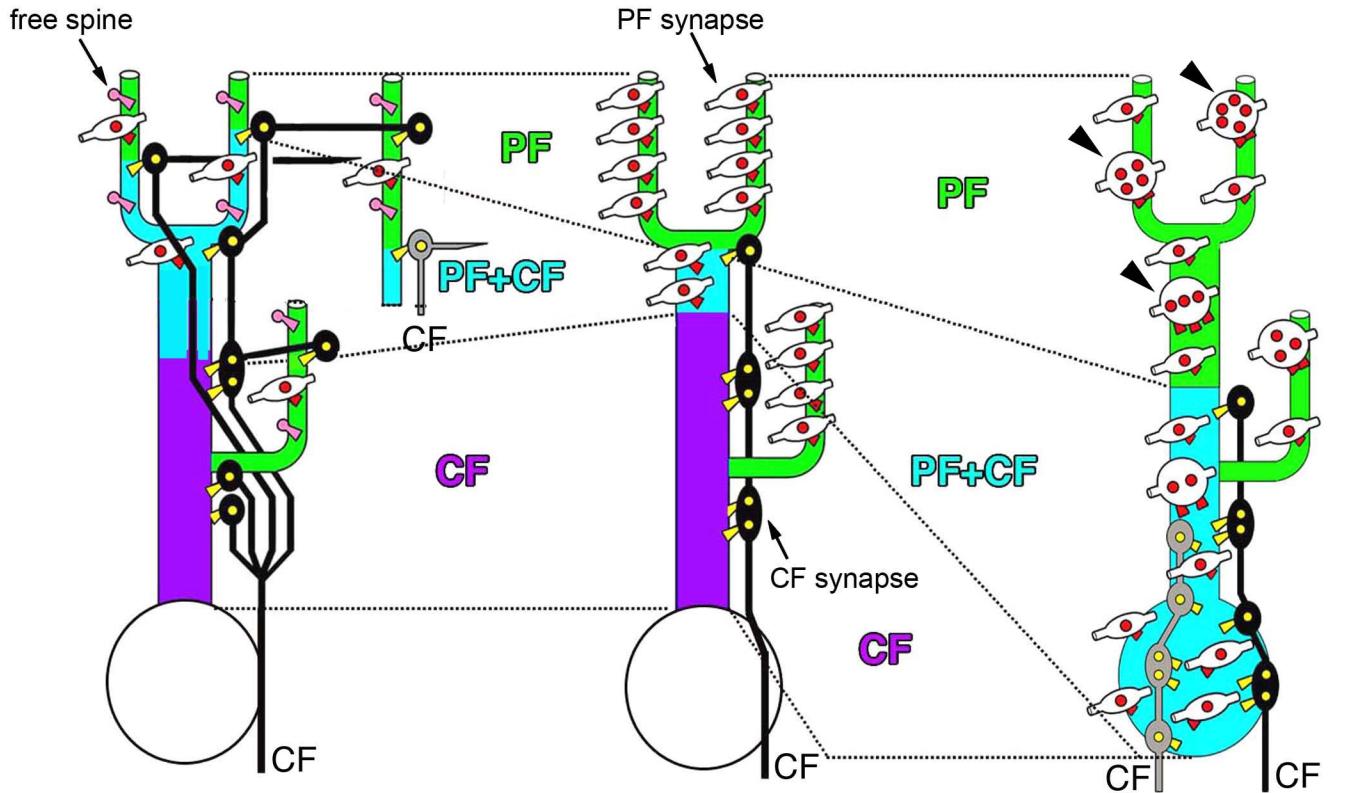


Early Phase



Late Phase





GluR δ 2 KO
Cbln1 KO

Wild type

CaV2.1 KO