CHAPTER

5

Cerebellar Circuits

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OUTLINE

5.1 OVERVIEW OF THE MICROCIRCUIT IN THE CEREBELLAR CORTEX

5.1.1 Cell Types and Afferent Fibers

The cerebellum consists of the cortex and the centrally located deep cerebellar nuclei (DCN). The cerebellar cortex exhibits a characteristic trilaminar structure composed of the molecular layer, Purkinje cell (PC) layer, and granular layer. In its mediolateral extent, the cerebellar cortex is divided into three longitudinal regions: vermis (medial cerebellum), paravermis (intermediate cerebellum or pars intermedia), and hemisphere (lateral cerebellum). Each of these regions is folded into lobules. The DCN also have three divisions: the medial (fastigial), interpositus (globose and emboliform), and lateral (dentate) nuclei, each of which is connected topographically with the vermis, paravermis, and hemisphere, respectively. Cerebellar neurons with distinct cytological and neurochemical properties reside in specific layers and sites of the cerebellum. They are connected with each other and also with specific brain regions outside the cerebellum (Figure 5.1).

PCs are the sole output neurons of the cerebellar cortex. The somata of PCs are aligned in the PC layer. PCs extend well-arborized dendrites in the molecular layer and project γ-aminobutyric acid (GABA)ergic axons to DCN and vestibular nuclei. There are two distinct excitatory afferents to the cerebellum, that is, climbing fibers (CFs) and mossy fibers (MFs; Palay and Chan-Palay, 1974). In adulthood, each PC is innervated by a single

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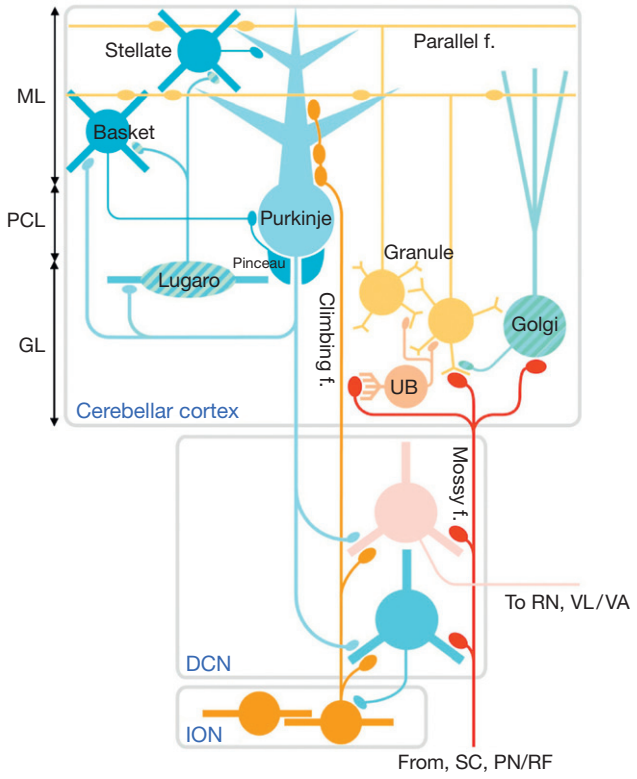

FIGURE 5.1 Neuronal component and synaptic wiring diagram of the cerebellum. Neurons painted with warm colors (yellow, orange, and pink) represent excitatory neurons, while those with cold colors (blue, light blue, and green) indicate inhibitory neurons. DCN, deep cerebellar nuclei; GL, granular layer; ION, inferior olivary nucleus; ML, molecular layer; PCL, Purkinje cell layer; UB, unipolar brush cell. PN, pontine nuclei; RF, reticular formation; RN, red nucleus; SC, spinal cord; VL/VA, ventral lateral/anterior thalamic nucleus.

CF originating from the inferior olive of the contralateral medulla oblongata (Eccles et al., 1966). Each CF forms hundreds of synapses by twisting around the proximal dendritic compartment. Therefore, activation of CFs causes strong depolarization of PC dendrites, triggers regenerative 'Ca^{2+} spikes' due to activation of voltage-gated Ca^{2+} channels (VDCCs) in PC dendrites (Miyakawa et al., 1992), and generates characteristic 'complex spikes' in the PC soma (Eccles et al., 1966) that are composed of a fast somatic Na^{+} action potential followed by slow dendritic Ca^{2+} spikes. In contrast, MFs originating from various extracerebellar regions, such as the spinal cord, pontine nuclei, and reticular formation, convey motor and sensory information to the distal dendritic compartment of PCs through parallel fibers (PFs), the axons of granule cells (GCs; Ito, 1984). Approximately, 10^5–10^6 PFs innervate a given PC, while each PF forms only one or two synapses onto individual PCs (Napper and Harvey, 1988). Thus, excitation of a single PF only weakly depolarizes PC dendrites, and about 50 GCs should fire synchronously to generate a single Na^{+} action potential (called 'simple spike') in the PC soma (Barbour, 1993). PFs also excite two types of GABAergic interneurons in the molecular layer, basket cells, and stellate cells. Although these two types of interneuron share similar features (Sultan and Bower, 1998), they differ in short-term plasticity (Bao et al., 2010), gene expression (Schilling and Oberdick, 2009), and most importantly, the target of projection. Basket cell axons innervate the soma and surround the axon initial segment (AIS) of PCs, while stellate cells innervate dendrites of PCs (Palay and Chan-Palay, 1974). The specialized conical structure formed by basket cell axons around the AIS is called the pinceau formation (Ango et al., 2004).

In the granular layer, there are several types of interneurons, which do not project to PCs directly. Golgi cells, Lugaro cells, and globular cells are inhibitory interneurons with a dual glycinergic/GABAergic phenotype (Ottersen et al., 1988). Golgi cells extend dendritic trees radiating through the cerebellar cortex. They receive excitatory inputs from PFs in the molecular layer and from MFs and presumably also from CFs in the granular layer. Golgi cells, in turn, innervate GC dendrites in the cerebellar glomeruli, thus providing both feedforward and feedback inhibition to GCs. Golgi cells also receive inhibitory inputs presumably from other Golgi cells, basket cells, stellate cells, and Lugaro cells. Lugaro cells are fusiform inhibitory interneurons lying just beneath the PC layer. They receive GABAergic inputs from axon collaterals of PCs and project axons to the molecular layer to innervate basket and stellate cells, thus providing feedback inhibition to PCs through these molecular layer interneurons (Laine and Axelrad, 2002). Globular cells have globular somata at variable depths in the granular layer and are thought to be a subtype of Lugaro cells (Laine and Axelrad, 2002). Lugaro cells and globular cells are neurochemically distinguished from Golgi cells; the former express calretinin, while most of the latter express mGluR2 and neurogranin (Simat et al., 2007). Unipolar brush cells are excitatory interneurons enriched in the granular layer of the vestibulocerebellum. They are characterized by single short dendrites terminating with a brush of dendrites, which engulf one or two rosettes of glutamatergic and cholinergic MFs. They innervate dendrites of other unipolar brush cells and GCs, thus regarded as an intermediate component that amplify excitatory drives of MFs on to GCs (Mugnaini and Floris, 1994).

DCN neurons receive excitatory inputs from collaterals of MFs and CFs, and inhibitory inputs from PC axons. Thus, information from the cerebellar cortex is integrated with direct inputs from MFs and CFs in DCN neurons. In the DCN, GABAergic neurons are either local interneurons or projection neurons targeting their axons to the inferior olive (De Zeeuw et al., 1988), while non-GABAergic neurons (presumably glutamatergic) project to the rest of the brain, including the red nucleus

and the thalamus. Thus, the DCN is in the key position of the cerebellar system and the source of cerebellar output.

5.1.2 Generation of Neurons that Constitute Microcircuits Related to PCs

All GABAergic neurons in the cerebellum originate from Ptf1a-expressing cells in the ventricular zone (Hoshino et al., 2005). Of these, projection neurons, that is, PCs and DCN neurons projecting to the inferior olive, are specified within the ventricular zone at the onset of cerebellar neurogenesis in early prenatal life (Altman and Bayer, 1997; Miale and Sidman, 1961). GABAergic interneurons also derive from the ventricular zone, but the progenitors continue to proliferate in the prospective white matter up to postnatal development (Zhang and Goldman, 1996). Phenotypic specification and diversity of GABAergic interneurons appear to be created by instructive cues provided by the microenvironment of the prospective white matter (Leto et al., 2009). On the other hand, the rhombic lip generates all progenitors of glutamatergic cerebellar neurons which then migrate via different pathways. Glutamatergic neurons in the DCN migrate to the nuclear transitory zone before descending to the prospective DCN (Fink et al., 2006). GC precursors first migrate to the cerebellar surface and form the external granular layer. There, they continue to proliferate during the postnatal period, and then descend to the internal granular layer (Rakic, 1971). Unipolar brush cells migrate to their destination through developing white matter (Englund et al., 2006).

5.1.3 Compartmentalization of the Cerebellum

Although the basic cellular composition and wiring diagram are uniform across the cerebellum, longitudinal organization of the cerebellar cortex has been demonstrated using anatomical, physiological, and molecular mapping techniques (Apps and Hawkes, 2009). Longitudinal cerebellar zones have been defined anatomically by cholinesterase labeling in the white matter and topographic projection of CFs and PC axons (Voogd and Ruigrok, 2004). Longitudinal zones are further divided into smaller units called microzones, based on high synchrony of complex spike activity (Llinas and Sasaki, 1989; Sugihara et al., 1993) and Ca^{2+} spikes (Mukamel et al., 2009; Schultz et al., 2009). Each microzone is ~500 μm in width, stable across behavioral states, and has a sharp boundary with the neighboring microzones (Mukamel et al., 2009). This synchrony is based on electrical coupling of nearby olivary neurons through dendrodendritic gap junctions (Llinas et al., 1974; Sotelo et al., 1974) and topographical olivocerebellar projections, that is, from given subregions of the inferior olive to specific longitudinal cortical zones (Sugihara et al., 2001).

Elaborate and fine compartmentalization can be recognized as cerebellar stripes by histochemistry for various molecules expressed in PCs. The best example of 'late' markers, which reveal the adult topography (postnatal day 15 onward), is aldolase C or zebrin II antigen (Hawkes and Leclerc, 1987), while 'early' markers of topography, such as calbindin and L7, typically reveal zones and stripes during perinatal development (embryonic day 13 (E13) to postnatal day 5(P5); Wassef et al., 1985)). These stripes are reproducible between individuals and conserved across species. Using a novel molecular marker, phospholipase Cβ4 (PLCβ4), which is continuously expressed in zebrin II-negative PCs from embryonic stage to adulthood (Nakamura et al., 2004; Watanabe et al., 1998), some stripes in the adult cerebellum have been shown to derive from two or more distinct embryonic clusters (Marzban et al., 2007). Importantly, studies with small tracer injections have shown that the topography of zebrin II expression pattern corresponds to that of the olivary projection to the cerebellar cortex and further to that of the olivary projection to the DCN and cerebellar cortical projection to DCN (Pijpers et al., 2005; Sugihara and Shinoda, 2004, 2007). These lines of evidence support the idea that the entire cerebellar system is formed by parallel assembly of an olivo–cortico–nuclear microcomplex (Ito, 1984).

5.2 DEVELOPMENT OF CF–PC SYNAPSES

5.2.1 Multiple Innervation of PCs by CFs in Early Postnatal Period

PCs undergo drastic changes in their morphology from late embryonic to early postnatal days (Armengol and Sotelo, 1991). PCs are born at the cerebellar ventricular zone at E10–E13 and migrate to form a multilayer below the molecular layer. At the end of radial migration, PCs exhibit bipolar shapes at E19–P0 ('simple and fusiform cell' by Armengol and Sotelo, 1991). Then, at P1–P3, new stem dendrites emerge from all aspects of the cell bodies, which confer the complex shapes of PCs ('complex-fusiform cell' by Armengol and Sotelo, 1991). From P3 to P6, such stem dendrites disappear by retraction of the long dendritic branches ('regressive–atrophic dendrites' by Armengol and Sotelo, 1991). In the meantime, PCs line up in a monolayer, which is completed by P5. PCs undergo explosive outgrowth of perisomatic protrusions that emerge in all directions from the cell bodies (the stage of 'stellate cells' by

Armengol and Sotelo, 1991). Upto P10, PCs extend single or double stem dendrites into the molecular layer, which grow and split into many dendritic branches, with simultaneous withdrawal of long somatic processes. The polarity of PCs is determined during this developmental period. From P10 to P15, the growth of the dendritic arbor occurs mainly in its lateral domain, whereas from P15 on, the dendritic growth occurs in the vertical plane and the height of the dendritic field reaches the adult level at P30.

After reaching the primitive cerebellum around E18, axons of inferior olivary neurons give rise to thick and thin collaterals (Wassef et al., 1992). This stage is called the 'creeper stage' (Chedotal and Sotelo, 1993) at which the 'CFs,' the thick collaterals of olivary axons, creep between immature PCs. At this stage, PCs have just completed their migration and are organized in a multilayer of 'simple and complex-fusiform cells'. Initially, each olivocerebellar axon forms about 100 'creeper' CFs (Sugihara, 2005). Then, the CF to PC synapse undergoes three distinct phases of postnatal development, which are described by Ramón y Cajal in his pioneering studies (Cajal, 1911): the 'pericellular nest' stage, the 'capuchon' stage, and the 'dendritic' stage. At the 'pericellular nest' stage, CFs surround the cell bodies of PCs and establish contacts with the abundant pseudopodia stemming from the soma and form a plexus on the lower part of the PC soma. At this stage, PCs have many perisomatic protrusions that emerge in all directions from the cell bodies, and therefore, this stage is called the phase of 'stellate cells' (Armengol and Sotelo, 1991). Among the 100 'creeper' CFs of each olivocerebellar axon, only around 10 can develop 'pericellular nests.' The 'capuchon' stage is characterized by the displacement of the plexus of CF collaterals to the apical portion of PC somata and main dendrites. Finally, at the 'dendritic' stage, CFs undergo translocation to growing PC dendrites and expanding their innervation territories.

Earlier electrophysiological studies on juvenile rats *in vivo* showed that stimulation to the inferior olive after P3 elicits CF-mediated responses in PCs (Crepel, 1971). However, in contrast to the all-or-none nature of CF responses in the adult animals, the responses of juvenile PCs are graded in parallel with the increase in the stimulus strength (Crepel et al., 1976). This was the first evidence that PCs are innervated by multiple CFs in early postnatal development. Later extensive studies *in vivo* revealed that both the percentage of PCs innervated by multiple CFs and the average number of CFs innervating individual PCs decrease with postnatal development and that most PCs become singly innervated by CFs (Crepel et al., 1981; Mariani and Changeux, 1981). These results clearly indicate that developmental elimination of redundant CF inputs occurs during postnatal development.

5.2.2 Functional Differentiation of Multiple CFs

Multiple CFs initially form functional synapses on the PC soma at around P3 (Chedotal and Sotelo, 1993; Morando et al., 2001). When recorded from PCs in cerebellar slices at this developmental stage, excitatory postsynaptic currents (EPSCs) elicited by stimulating multiply-innervating CFs are much smaller than those of mature CFs (Bosman et al., 2008; Hashimoto and Kano, 2003, 2005; Ohtsuki and Hirano, 2008; Scelfo and Strata, 2005). Therefore, CF inputs become stronger, while redundant CFs are eliminated during postnatal development.

Changes in the relative synaptic strengths of multiple CFs innervating the same PC have been systematically studied during postnatal development (Hashimoto and Kano, 2003) by recording CF-mediated EPSCs in PCs from cerebellar slices of mice aged P2–P21. This systematic study showed that more than five discrete CF-EPSCs with similar amplitudes are recorded in PCs from neonatal mice around P3 (Figure 5.2(a), ∼P3). In contrast, in the second postnatal week, PCs with multiple CF-EPSCs have one large CF-EPSC and a few small CF-EPSCs (Figure 5.2(a), ∼P7 and ∼P12). These results indicate that synaptic strengths of multiply-innervating CFs are relatively uniform in neonatal mice, and one CF is selectively strengthened during postnatal development (Bosman et al., 2008; Hashimoto and Kano, 2003, 2005). Quantitative assessments 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 (Figure 5.2(b); Hashimoto and Kano, 2003). These electrophysiological data are consistent with the morphological observation that the innervation pattern of CFs over PCs drastically changes during this postnatal period in rats (Sugihara, 2005). At P4, CFs have many creeping terminals in the PC layer and their swellings do not aggregate at particular PC somata (creeper type). Then, from P4 to P7, CFs surround several specific PC somata and form aggregated terminals on them (nest type; Sugihara, 2005).

There are clear differences in electrophysiological properties between EPSCs elicited by the strongest CF input and those by other weaker inputs. Transient rises of glutamate concentration in the synaptic cleft are significantly higher after stimulation of the strongest CF than the 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 vesicle released simultaneously to a given postsynaptic site from the corresponding presynaptic release site) is higher for the strongest CF than for the weaker CFs. Further electrophysiological examination suggested that

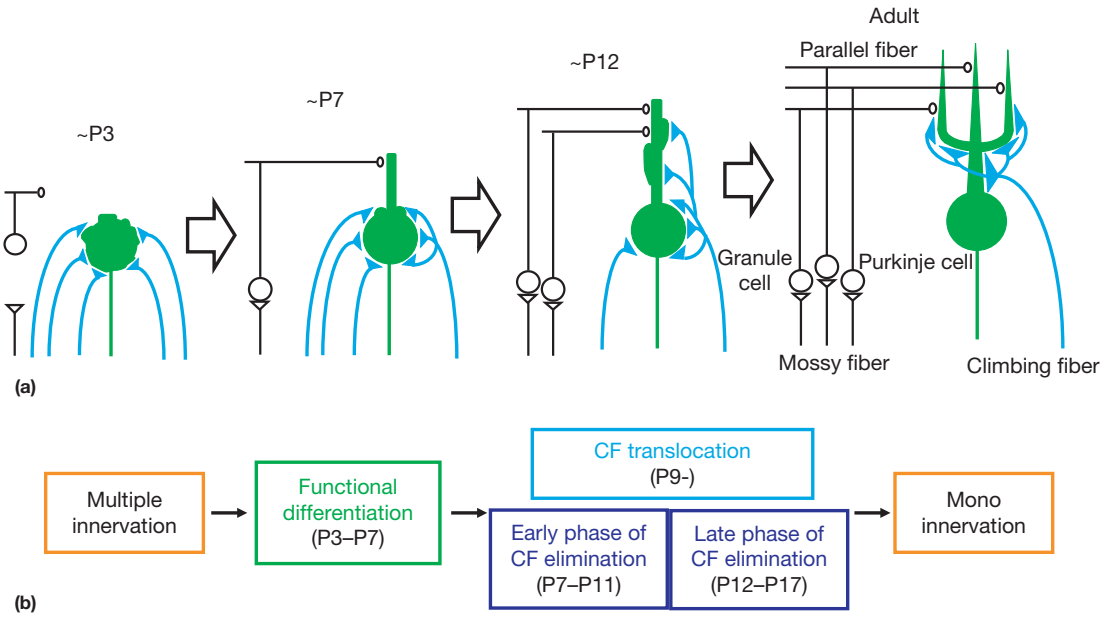

FIGURE 5.2 Postnatal development of CF–PC synapses. (a) Diagrams of CF–PC synapses at four representative stages of postnatal development in mice. (b) Four distinct phases in postnatal development of CF–PC synapses. *Reproduced with permission from* European Journal of Neuroscience *34, 1697–1710, with permission*.

the number of release sites facing a narrow postsynaptic PC region is larger in the strongest CF than in weaker CFs (Hashimoto and Kano, 2003).

5.2.3 Dendritic Translocation of Single CFs

Morphological evidence indicates that the site of CF innervation of PC changes from soma to dendrite during early postnatal development, a phenomenon known as 'CF translocation' (Altman and Bayer, 1997). The relationship between the selective strengthening of single CFs and CF translocation was investigated by using both electrophysiological and morphological techniques (Hashimoto et al., 2009a). The location of synapses along the somatodendritic domains of PCs can be estimated 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 on each PC has just been completed, there is no significant difference in the distribution of the rise times (i.e., time from the onset to the peak) of qEPSCs for the strongest compared with the weaker CFs. Since the rise time of qEPSCs is proportional to the distance from the synaptic sites to the somatic recording site (Roth and Hausser, 2001), synapses of the strongest and weaker CFs are thought to be located on the soma at around P7 (Figure 5.2(a), ~P7). At P9–P10, the incidence of qEPSCs with slow rise times was more frequent for the strongest than for the weaker CFs, suggesting the initiation of CF translocation (Figure 5.2(b)). The difference in the distribution of qEPSC rise times for the strongest compared with the weaker CFs becomes larger from P11 to P14. While the incidence of qEPSCs with slow rise times becomes more frequent for the strongest CFs with age, the qEPSC rise times for weaker CFs remain almost unchanged from P9 to P14. These electrophysiological data collectively indicate that (1) synaptic competition among multiple CFs occurs on the soma before P7 (Figure 5.2(a), ~P3 and ~P7, Figure 5.2(b)), (2) only the strongest CF ('winner' CF) starts to translocate to dendrites at P9 and the translocation continues thereafter (Figure 5.2(a), ~P12, Figure 5.2(b)), and (3) synapses of the weaker CFs ('loser' CFs) remain around the soma (Figure 5.2(a), ~P12).

Morphological data are consistent with these electrophysiological observations. When subsets of CFs are labeled by an anterograde tracer, biotinylated dextran amine (BDA), injected into the inferior olive, pericellular nests with extensive branching of CFs are observed at P7, P9, and P12. At P7, in spite of the presence of immature stem dendrites in PCs, CFs innervate the soma and spare the dendrites (Figure 5.3(a)). Dendritic innervation of CFs starts at P9 (Figure 5.3(b)) and at P12, and thereafter, the territory of innervation extends progressively along the PC dendrites (Figure 5.3(c) and 5.3(d)).

5.2.4 Early Phase of CF Synapse Elimination

Detailed assessment of the postnatal development of CF innervations in mouse cerebellar slices demonstrates that there is no significant reduction in the average number of CFs per PC from P3 to P6 when functional

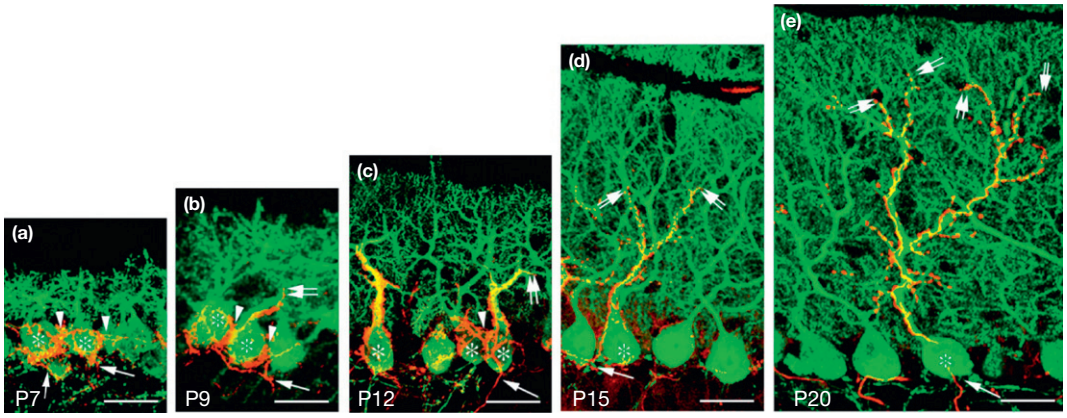

FIGURE 5.3 Developmental profile of CF innervations from perisomatic nest stage to peridendritic stage. (a–e) Fluorescent labeling of CFs with BDA (red) and PCs with calbindin antibody (green) at respective postnatal days. *Reproduced from Hashimoto K, Ichikawa R, Kitamura K, Watanabe M, and Kano M (2009) Translocation of a 'winner' climbing fiber to the Purkinje cell dendrite and subsequent elimination of 'losers' from the soma in developing cerebellum.* Neuron *63: 106–118, with permission.*

differentiation of multiple CFs occurs (Hashimoto et al., 2009b). The value then decreases progressively from P6 to around P15 (Hashimoto et al., 2009b; Scelfo and Strata, 2005). CF synapse elimination therefore does not proceed in parallel with functional differentiation of multiple CFs but starts after the strengthening of single CFs to individual PCs. Crepel et al. (1981) showed that elimination of surplus CFs consisted of two distinct phases, the early phase up to around P8 and the late phase from around P9 to P17 (Crepel et al., 1981). The early phase occurs normally in animals with mild X-irradiation to the cerebellum during the early postnatal period, which causes selective loss of GCs and PFs while leaving PCs intact. In marked contrast, the late phase is severely impaired by inhibiting GC production by X-irradiation. This study indicates that the early phase of CF synapse elimination is independent of PF–PC synapse formation, whereas the late phase is critically dependent on it. However, since the animal models with 'hypogranular' or 'agranular' cerebella often have abnormalities of cerebellar development 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 the glutamate receptor δ2 subunit (GluRδ2 or GluD2) demonstrates that there are two distinct phases of CF synapse elimination. GluRδ2 is richly expressed in PCs and its deletion causes impairment of PF–PC synapse formation leading to reduction of PF–PC synapse number to about half of that in wild-type mice. In spite of the severe impairment of PF synapse formation, GluRδ2 deletion does not significantly affect the laminar structure of the cerebellum and morphology of the PC and its dendritic tree (Kashiwabuchi et al., 1995; Kurihara et al., 1997). In GluRδ2 knockout mice, the average number of CFs innervating each PC is similar to that of control mice from P5 to P11. However, the value is significantly larger than that of control mice from P12 to P14. Thus, CF synapse elimination in mice can be classified into two distinct phases, namely, the 'early phase' from P6 to around P11 which is independent of PF–PC synapse formation and the 'late phase' from around P12 and thereafter which requires normal PF–PC synapse formation (Figure 5.2(b); Hashimoto et al., 2009b).

Molecular mechanisms of the early phase of CF synapse elimination remain largely unknown. However, patterns of CF activity have been reported to influence the early phase of CF synapse elimination. Andjus et al. disrupted the normal activity pattern of CFs in rat at P9–P12 by administration of harmaline, which induced synchronous activation of inferior olive neurons (Andjus et al., 2003). This treatment caused persistent multiple CF innervations of PCs in rats at P15–P87. Furthermore, a recent report strongly suggests that PC activity is crucial for CF synapse elimination. Lorenzetto et al. (2009) generated transgenic mice that expressed a chloride channel-YFP fusion protein specifically in PCs to suppress their excitabilities. In these mice, the expression of chloride channel was observed in PCs during the 'early phase' at P9, and multiple CF innervations persisted up to P90. Therefore, perturbation of PC activity is considered to cause impairment of the 'early phase' of CF synapse elimination. In addition, insulin-like growth factor I (IGF-1) is reported to be involved in CF synapse elimination from P8 to P12 (Kakizawa et al., 2003). IGF-1 is thought to enhance the strengths of CF synapses and promote their survival, whereas the shortage of IGF-1 appears to impair the development of CF synapses (Kakizawa et al., 2003). In addition, Sherrard et al. reported recently that the active, phosphorylated form of full-length TrkB, a receptor for

brain-derived neurotrophic factor (BDNF), fell around the onset of the early phase of CF synapse elimination. In contrast, the expression of the truncated form, which acts as a negative regulator of TrkB signaling, rose at the same developmental stage (Sherrard et al., 2009). This finding suggests that decrease in TrkB signaling might permit the elimination of surplus CF synapses, although TrkB signaling appears to be involved in the 'late phase' of CF synapse elimination presumably through the maturation of GABAergic inhibitory synapses (see Section 5.2.5).

Morphological data indicate that CFs that undergo dendritic translocation keep their synapses on the PC soma during the second postnatal week. In contrast, synaptic terminals of the weaker CFs are confined to the soma and the basal part of the primary dendrite. The characteristic pericellular nest consists of somatic synapses originating from collaterals of a single predominant CF and from weaker CFs and thus represents multiple CF innervation of PCs (Hashimoto et al., 2009a). Therefore, CF synapse elimination is thought to be a process of nonselective pruning of perisomatic synapses, which spares dendritic synapses of a single predominant CF and leads to monoinnervations of that CF (Hashimoto et al., 2009a).

Hashimoto et al. (2011) have recently reported that the P/Q-type VDCC expressed in PCs drives the early phase of CF synapse elimination (Hashimoto et al., 2011). They generated mice with PC-selective deletion of $Ca_V2.1$, a pore-forming subunit of the P/Q-type VDCC that constitutes the major Ca^{2+} current component in PCs (PC-$Ca_V2.1$ KO mice; Mintz et al., 1992; Stea et al., 1994). Although initial CF to PC synapse formation appears normal at around P4 in PC-$Ca_V2.1$ KO mice, subsequent CF synapse development and elimination are severely impaired. First, biased strengthening of a single CF input in each PC from P5 to P7 is absent in PC-$Ca_V2.1$ KO mice, and multiple CF inputs equally become larger by about fourfold. Second, more than one CF undergoes translocation to dendrites in PC-$Ca_V2.1$ KO mice. Third, CF synapse elimination is severely impaired in PC-$Ca_V2.1$ KO mice until around P12 (Hashimoto et al., 2011). Global $Ca_V2.1$ KO mice have essentially the same defects in CF synapse development and elimination as PC-$Ca_V2.1$ KO mice (Hashimoto et al., 2011; Miyazaki et al., 2004). These results indicate that Ca^{2+} influx through P/Q-type VDCC into PCs is essential for selective strengthening of a single CF input in each PC, dendritic translocation of the strengthened CF, and the early phase of CF synapse elimination.

5.2.5 Late Phase of CF Synapse Elimination

In GluRδ2 knockout mice, CFs invade into the distal dendrites and form ectopic synapses there (Hashimoto et al., 2001; Ichikawa et al., 2002). These ectopic CF synapses appear around P10 when PF synapse formation and PC dendritic arborization occur most vigorously. The similar type of multiple CF innervation is also found in a mutant mouse deficient in cbln1 in which PF to PC synapse formation is severely impaired (Hirai et al., 2005). These results indicate that PFs compete for the innervation territory with CFs during development and play a role in restricting CF innervation to proximal dendrites (Figure 5.4). The details of this phenomenon will be described in Section 5.3.3.

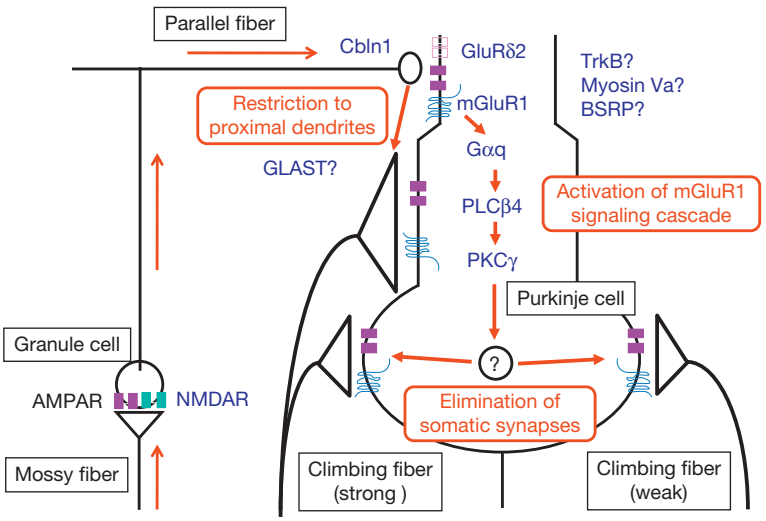

FIGURE 5.4 Mechanisms underlying the late phase of CF synapse elimination. PF–PC synapses play two distinct roles in the late phase. PF synapses are formed and maintained on distal dendrites of PCs through the interaction of Cbln1 and GluRδ2. First, PF synapses occupy the postsynaptic sites on the distal dendrites and confine the CF innervation sites to the proximal dendrites (restriction to proximal dendrites). Second, neural activity along the pathway of mossy fiber, GC, and PF involving N-methyl-D-aspartate (NMDA) receptor at MF to GC synapses drives mGluR1 to PKCγ signaling cascades in PCs (activation of mGluR1 signaling cascade). The signaling molecules downstream of PKCγ are currently unknown, but this eventually leads to elimination of somatic CF synapses including those of weak CFs and of somatic collaterals of strong CF (elimination of somatic synapses). TrkB, myosin Va, brain-specific receptor-like protein (BSRP), and glutamate-aspartate transporter (GLAST) are also involved in the late phase of CF synapse elimination, but details of the mechanisms of their actions in CF synapse elimination are unknown. *Modified from Kano, M., Hashimoto, K., Tabata, T., 2008. Type-1 metabotropic glutamate receptor in cerebellar Purkinje cells: a key molecule responsible for long-term depression, endocannabinoid signalling and synapse elimination. Philosophical Transactions of the Royal Society B 363, 2173–2186.*

Another role of PF synapses is to activate type 1 metabotropic glutamate receptor (mGluR1) and its downstream signaling cascades in PCs to drive the process of CF synapse elimination. It is shown that the mutant mouse deficient in mGluR1 is impaired in CF synapse elimination (Kano et al., 1997; Levenes et al., 1997). Mice deficient in signaling molecules downstream of mGluR1, that is, $G_{\alpha q}$, PLCβ4, and PKCγ, are also impaired in CF synapse elimination (Hashimoto et al., 2000; Kano et al., 1995, 1998; Offermanns et al., 1997). Electrophysiological examination of CF innervation following postnatal development demonstrates that the regression of CF synapse normally occurs during the first and second postnatal weeks in all of the four mouse strains. However, these mice display abnormality in CF synapse elimination during the third postnatal week. These results suggest that the signaling cascade from mGluR1 to protein kinase C (PKC)γ is essential for the late phase of CF synapse elimination, but it is dispensable for the early phase of CF synapse elimination (Figure 5.2(b)). Importantly, the formation and function of PF to PC synapses are normal in these mutant mice. Therefore, the impaired CF synapse elimination is not caused secondarily by the defect in PF synaptogenesis.

The defect in the CF synapse elimination in the mGluR1-deficient mouse is restored in the mGluR1-rescue mice in which mGluR1a has been introduced specifically into PCs (Ichise et al., 2000). Regression of CF synapses is impaired in mice by PC-specific expression of a PKC inhibitor peptide (De Zeeuw et al., 1998). Furthermore, the distribution of multiply-innervated PCs in the cerebellum of PLCβ4 knockout mice exactly matches that of the PCs with predominant expression of PLCβ4 in the wild-type mouse cerebellum (Kano et al., 1998). These lines of evidence clearly indicate that the signaling from mGluR1 to PKCγ in PCs but not other cell types plays a central role in CF synapse elimination.

The mGluR1 signaling required for the late phase of synapse elimination is 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 N-methyl-D-aspartate (NMDA) receptors within the cerebellum 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 present at either PF or CF synapses onto PCs during second and third postnatal weeks, but they are abundantly expressed at MF to GC synapses (Kakizawa et al., 2000). Therefore, the chronic blockade of NMDA receptors within the cerebellum should affect MF to GC transmission. These results suggest that neural activity along MF–GC–PF–PC pathway and subsequent activation of mGluR1 are prerequisite for the late phase of CF synapse elimination (Figure 5.4; Kakizawa et al., 2000).

Besides the mGluR1 signaling in PCs, a neurotrophin receptor, TrkB (Bosman et al., 2006; Johnson et al., 2007), a motor protein, myosin, Va (Takagishi et al., 2007), a glutamate transporter, glutamate-aspartate transporter (GLAST) (Watase et al., 1998), and a novel brain-specific receptor-like protein family, brain-specific receptor-like protein (Miyazaki et al., 2006), are also involved in CF synapse elimination. Since genetic or pharmacological deletion of these molecules in mice impairs CF synapse elimination in the second postnatal week, these signaling cascades are thought to be involved in the 'late phase' of CF synapse elimination. The involvement of a neurotrophin receptor, TrkB, is especially interesting, because TrkB signaling is required for normal development of GABAergic innervations of PCs. In TrkB knockout mice, the number of GABAergic synapses is reduced and the inhibitory postsynaptic currents are prolonged (Bosman et al., 2006), which suggest the $GABA_A$ receptors do not undergo the normal α3 to α1 subunit switching (Takayama and Inoue, 2004). It is therefore possible that normal development of GABAergic inhibitory synapses onto PCs is prerequisite for the late phase of CF synapse elimination (Nakayama et al., 2012). As for other molecules potentially involved in the late phase of CF synapse elimination, 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 what stage of postnatal development the impairment occurs (Ribar et al., 2000). It is also reported that null mutant mice deficient in α-calcium/calmodulin-dependent protein kinase II (CaMKIIα) display multiple CF innervations at P21–P28, but this phenotype disappears in adulthood (Hansel et al., 2006), suggesting that CaMKIIα deficiency delays but does not prevent CF synapse elimination.

During synapse elimination in the neuromuscular junction, bulb-shaped tips of retreating motor axons and the axon fragments ('axozomes') are engulfed by Schwann cells (Bishop et al., 2004). These axon bulbs, axozomes, and Schwann cell cytoplasm are often positively stained with Lysotracker Red, a marker for the lysosomes and late endosomes of living cells, suggesting axonal digestion through autophagy and subsequent heterophagy by Schwann cells (Song et al., 2008). It is also reported that Lysotracker-positive structures surrounding PCs, which are presumed to be within Bergmann glia, are abundant during the second and third postnatal weeks (Song et al., 2008). This result suggests that retreating CF axons might be digested in a manner similar to the retreating motor axons at neuromuscular junction.

The current model for the mechanisms underlying the late phase of CF synapse elimination is illustrated in Figure 5.4. First, PF synapses confine the CF innervation territories to proximal dendrites of PCs (Figure 5.4). Second, PF activity involving NMDA receptor at MF to

GC synapses drives mGluR1 to PKCγ signaling cascades in PCs (Figure 5.4), which leads to nonselective elimination of somatic CF synapses (Figure 5.4). The signaling molecules downstream of PKCγ are currently unknown. The molecular mechanisms underlying morphological elimination of the weaker CFs are also unknown. Some mechanisms must convey transsynaptic retrograde signaling from PCs to weaker CFs.

A recent study using organotypic slice culture shows that CF synapse elimination occurs only during the critical period that depends on the maturation stage of postsynaptic PCs but not on presynaptic olivary neurons (Letellier et al., 2009). The authors cocultured immature or mature medulla containing the inferior olive with naïve or non-naïve PCs (i.e., PCs that have not undergone synapse elimination or those that have experienced synapse elimination, respectively). They found that multiple CF innervation was observed when either the PCs were naïve or CFs were immature. Interestingly, non-naïve PCs could not eliminate multiple CFs. These results suggest that CF synapse elimination during the critical period leaves indelible trace in PCs that prevents the elimination process from occurring in the later stage (Letellier et al., 2009).

5.3 DEVELOPMENT OF PF–PC SYNAPSES

5.3.1 Formation of PF–PC Synapses

PF–PC synapses increase in number and mature in the early postnatal period, concomitant with differentiation of GCs and growth of PC dendrites. During the first 10 days of a rodent's life, production and migration of GCs as well as growth of PC dendrites are slow (Altman and Bayer, 1997). In this period, the proliferative or outer zone of the external granular layer has constant thickness of four to five cells, while the depth of the premigratory or inner zone increases progressively (Altman and Bayer, 1997). In the premigratory zone, postmitotic GCs extend future PFs in the transverse plane, and then migrate downward along Bergmann fibers in the molecular layer (Altman and Bayer, 1997; Rakic, 1971). As a consequence, T-shaped axon of GCs differentiates, and horizontal beams of PFs become a part of the upper zone of the molecular layer. PC dendrites are immature, particularly in the upper zone of the molecular layer, where dendrites extend filopodium-like protrusions, PF–PC synapses are few in number, spines are often free of innervation, and coverage by Bergmann glia is incomplete (Kurihara et al., 1997; Yamada et al., 2000).

In the next ten postnatal days, PC dendrites grow dynamically, the bulk of GCs come into existence, and PF–PC synapses explosively increase in number (Takacs and Hamori, 1994). Moreover, almost all spines form synaptic contact with PF terminals, and PF–PC synapses are equipped with well-developed postsynaptic density and complete astroglial coverage (Kurihara et al., 1997; Spacek, 1985; Yamada et al., 2000). Analyses using agranular and hypogranular animal models, where PF–PC synaptogenesis is hindered by spontaneous gene mutation or postnatal X-ray irradiation, have demonstrated that PF synapse formation in PCs plays a critical role in the elongation, branching, and planar development of dendritic trees (Sotelo, 2004).

5.3.2 Stabilization and Maintenance of PF–PC Synapses

Our understanding of the molecular mechanism of PF synapse formation in PCs has been greatly advanced by molecular identification of GluRδ2 (Araki et al., 1993; Lomeli et al., 1993) and analyses of mutant mice defective in this gene *grid2* (GluRδ2-knockout mice and spontaneous *hotfoot* mutant mice; Guastavino et al., 1990; Kashiwabuchi et al., 1995). Like other ionotropic GluR subunits, GluRδ2 preserves three transmembrane domains (TM1, TM3, and TM4), reentrant hairpin loop (TM2) surrounding a channel pore, ligand-binding domains in the *N*-terminal region, and protein–protein interaction sites in the *C*-terminal region (Uemura et al., 2004; Yuzaki, 2004). However, GluRδ2 does not function as a glutamate-gated ion channel (Kakegawa et al., 2007a,b).

In the brain, GluRδ2 is expressed almost exclusively in PCs (Araki et al., 1993; Lomeli et al., 1993) and selectively localized at PF but not CF synapses (Landsend et al., 1997; Takayama et al., 1995). PCs in GluRδ2-defective mice display characteristic phenotypes mostly related to PF synapse structure and function, including reduction in the number of PF synapses per PC to about half of the wild-type mice (54% in control PCs; Kurihara et al., 1997), emergence of free spines lacking synaptic contact in the distal dendritic domain (37% of the total spines; (Ichikawa et al., 2002; Kurihara et al., 1997)), mismatching of pre- and postsynaptic specialization at PF synapses (Guastavino et al., 1990; Takeuchi et al., 2005), impaired long-term depression (LTD) at PF synapses (Kakegawa et al., 2008; Kashiwabuchi et al., 1995; Uemura et al., 2007), impaired motor learning (Kakegawa et al., 2008; Kishimoto et al., 2001), and severe ataxia (Guastavino et al., 1990; Kashiwabuchi et al., 1995). In a drug-inducible, PC-specific GluRδ2-knockout mouse strain, mismatched PF–PC synapses, free spines, and motor discoordination are induced and exacerbated in the adult cerebellum, concomitant with a decrease in GluRδ2 protein (Takeuchi et al., 2005). Furthermore, expression of the *N*-terminal domain of GluRδ2 in human embryonic kidney cells induces presynaptic differentiation (Uemura

and Mishina, 2008) and its viral transfer to adult GluRδ2-knockout mice rapidly restores PF synapse formation and motor discoordination (Kakegawa et al., 2009). Thus, GluRδ2 strengthens and regulates the connectivity of PF–PC synapses in both the developing and adult cerebella. On the other hand, the last seven amino acids known as the T-site, which binds to various PDZ domain-containing proteins including postsynaptic density (PSD)-93, PTPMEG, delphilin, nPIST, and synaptic scaffolding molecule are essential for cerebellar LTD and motor learning (Kakegawa et al., 2008; Uemura et al., 2007).

Cbln1 or precerebellin was originally identified as a precursor of PC-specific peptide cerebellin (Slemmon et al., 1984). However, C-terminal two-thirds of Cbln1 shares significant structural similarity with the globular domain of complement C1q chain (Urade et al., 1991), and the full-length Cbln1 is released into the culture medium as a hexameric complex (Bao et al., 2005). Thus, Cbln1 now belongs to the C1q/tumor necrosis factor superfamily. Of four members (Cbln1–4), Cbln1 is highly expressed in cerebellar GCs together with Cbln3 (Hirai et al., 2005; Miura et al., 2006), exists as Cbln1 homomeric and Cbln1/3 heteromeric complexes (Iijima et al., 2007; Pang et al., 2000), and selectively accumulates in the synaptic cleft facing PF terminals, but not CF terminals, in PCs (Iijima et al., 2007; Miura et al., 2009). Cbln1-knockout mice show characteristic phenotypes similar to, or even severer than, GluRδ2-knockout mice (Hirai et al., 2005). In PCs of Cbln1-knockout mice, 78% of the spines are free of innervation and 14% have mismatching in pre- and postsynaptic differentiation (Figure 5.5). Hence, only 8% of spines in Cbln1-knockout mice establish normal matched synapses with PF terminals. LTD at PF–PC synapses and motor coordination is also impaired. When recombinant Cbln1 is applied to the subarachnoid space of adult Cbln1-knockout mice, only a single injection can rapidly restore PF–PC synapse structure and function, and cerebellar ataxia (Ito-Ishida et al., 2008). These common phenotypes in GluRδ2- and Cbln1-knockout mice and their rapid rescue by genetic or molecular supplementation are explained by the fact that transsynaptic interaction of postsynaptic GluRδ2 and presynaptic neurexins is mediated by Cbln1. Thus, through this unique interaction, Cbln1 acts as a bidirectional synaptic organizer for both pre- and postsynaptic components at PF–PC synapses (Matsuda et al., 2010; Uemura et al., 2010).

The strengths of PF synapses, but not CF synapses, in PCs are selectively decreased when postsynaptic mGluR1 or inositol 1,4,5-trisphosphate (IP_3) signaling is chronically inhibited, when PF activity is inhibited by suppressing NMDA receptor-mediated inputs to GCs or when antibody against BDNF is applied *in vivo* (Furutani et al., 2006). The weakening is due to reduction of glutamate release probability from PFs. The weakening of PF–PC synaptic strength is reversed by

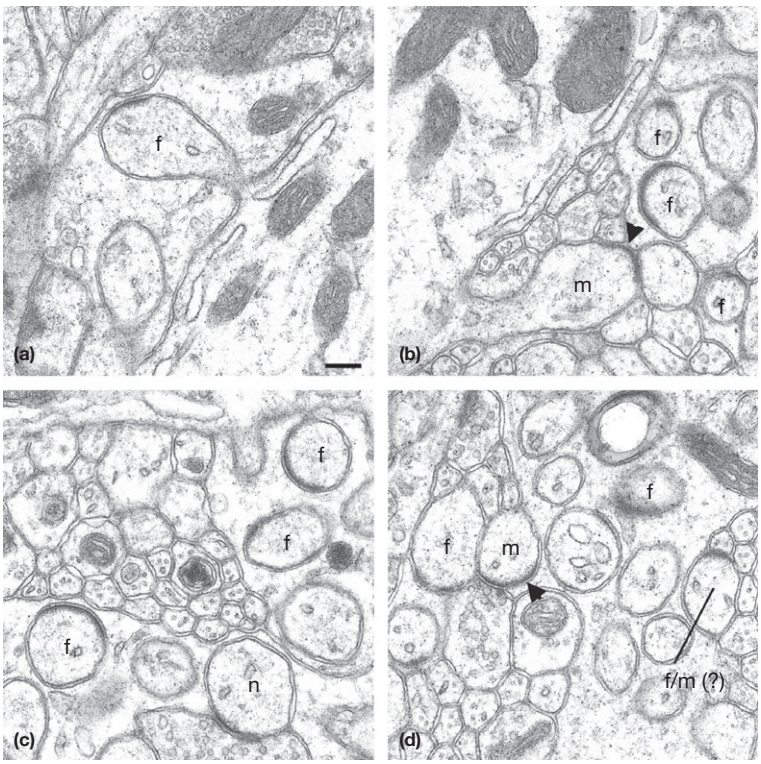

FIGURE 5.5 Electron micrographs of free spines (f) and mismatched synapses (m) in Cbln1-knockout mice. Free spines are thoroughly enwrapped by the lamellate processes of Bergmann glia. *Arrowheads* indicate the portion of the PSD that is not opposed by the presynaptic active zone. Normal matched synapses (n) are very rare in this mutant. In (d), the presence of postsynaptic density with no presyanptic terminal differentiation suggests that the spine with such postsynaptic density is either free or mismatched spine (hence labeled "f/m(?)." Scale bar = 0.2 μm. *Reproduced from Watanabe, M., Molecular mechanisms governing competitive synaptic wiring in cerebellar Purkinje cells.* Tohoku Journal of Experimental Medicine *214, 2008:175–190, with permission.*

in vivo application of BDNF. These results suggest that postsynaptic mGluR1 activation and the following IP_3 signaling maintain presynaptic function through BDNF at PF–PC synapses (Furutani et al., 2006). In this regard, it is interesting to note that free spines on PC dendrites emerge in the spontaneous ataxic mutant rigoletto (*rig*) (also known as waddles; *wdl*), which is caused by a 19-bp deletion in the exon 8 of carbonic anhydrase-related protein *Car8* (Hirasawa et al., 2007). Car8 is known to bind to IP_3 receptor and reduce its affinity for IP_3 (Hirota et al., 2003). In future studies, it is important to investigate how Car8 modulates the mGluR1-IP_3 signaling and whether Car8 is involved in the GluRδ2–Cbln1–neurexin interaction, both of which regulate the connectivity of PF–PC synapses.

5.3.3 Heterosynaptic Competition Between PF and CF Inputs

The proximal compartment of PC dendrite appears smooth in contour due to low spine density and is innervated by single CFs. On the other hand, the distal compartment is made up of spiny branchlets studded with numerous spines and innervated by PFs (Figure 5.6, middle). Accumulated experimental evidence indicates that the construction of such excitatory synaptic organization stands on competitive equilibrium between CFs and PFs whose expansions are promoted by distinct mechanisms. Surgical, pharmacological, and genetic manipulations that shift this equilibrium can alter the organized innervations pattern.

Disruption of GluRδ2 gene in mice not only causes abnormal structure and function of PF synapses but also affects the mode of CF innervations (Ichikawa et al., 2002). In the molecular layer, CF branches are distributed in the inner four-fifths (84% of the molecular layer thickness) in control mice, whereas their distribution almost reaches the pial surface (95%) in GluRδ2-knockout mice. When the tracer-labeled CFs were followed from the soma to the tips of PC dendrites by serial electron microscopy, CF branches in GluRδ2-knockout mice extend distally and take over the free spines on the distal dendrites. Such aberrant extension occurs toward not only distal dendrites of the same PCs but also those of the neighboring PCs. The latter type of spine takeover results in multiple innervation of a PC by CFs of different neuronal origins. This anatomical evidence for multiple CF innervation is consistent with electrophysiological recording combined with Ca^{2+} imaging. In GluRδ2-knockout mice, a single strong CF elicits large EPSCs with a fast rise time and large Ca^{2+} transients over the entire dendritic tree, whereas weak CFs elicit small EPSCs with a slow rise time and small Ca^{2+} elevation that is confined to distal dendrites (Hashimoto et al., 2001). These findings indicate that GluRδ2 is essential for restricting CF innervation to the proximal dendritic compartment and thereby preventing multiple CF innervation at the distal dendritic compartment (Figures 5.4 and 5.6, right).

This mechanism is also active in the adult cerebellum. The ablation of GluRδ2 in adulthood also leads to progressive distal extension of ascending branches of CFs, and they aberrantly innervate distal dendrites of the target and neighboring PCs (Miyazaki et al., 2010). Furthermore, transverse branches of CFs, which are short motile collaterals forming no synapses in wild-type animals (Nishiyama et al., 2007), display aberrant mediolateral extension and innervate distal dendrites of neighboring and remote PCs. Consequently, many PCs are connected by single main CFs and surplus CFs that innervate small parts of the distal dendrites. Surplus CF-EPSCs with slow rise time and small amplitude also emerge progressively after GluRδ2 ablation. Therefore,

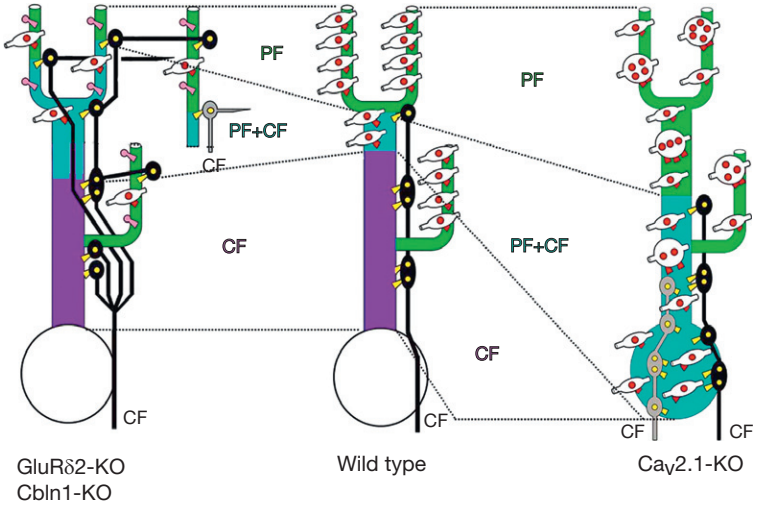

FIGURE 5.6 Summary diagram of molecular mechanisms for competitive synaptic wiring in PCs. Note that the climbing fiber (CF) and parallel fiber (PF) territories are reversed in mutant mice defective in GluRδ2/Cbln1 (left) and $Ca_V2.1$ (right). With both mechanisms, CF and PF territories are sharply segregated, and CF mono-innervation is established in wild-type animals (middle). *Reproduced from Watanabe, M., 2008. Molecular mechanisms governing competitive synaptic wiring in cerebellar Purkinje cells.* Tohoku Journal of Experimental Medicine *214, 2008:175–190, with permission.*

GluRδ2 is essential to keep CF mono-innervation in the adult cerebellum by suppressing aberrant invasion of CF branches to the territory of PF innervation.

In contrast, CF innervation is regressed but PF innervation expands to the proximal compartment, when surgical lesion to olivocerebellar projections is made in adult animals or activities in the cerebellar cortical neurons are blocked with the sodium channel blocker tetrodotoxin or with the synaptic scaffolding molecule AMPA) receptor antagonist 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo [f]quinoxaline-7-sulfonamide (Bravin et al., 1995; Cesa et al., 2007; Kakizawa et al., 2005). The latter change often accompanies hyperspiny transformation at the proximal dendritic compartment (Bravin et al., 1995; Cesa et al., 2007). Similar changes are reproduced in global knockout mice of $Ca_V2.1$, a pore-forming subunit of the P/Q-type VDCC (Miyazaki et al., 2004). In $Ca_V2.1$-knockout mice, hyperspiny transformation is induced at proximal dendrites and somata of PCs, and many of these ectopic spines are innervated by PF terminals. Conversely, the distribution of CFs is regressed to lower portions of the molecular layer, and they innervate spines from somata and basal dendrites. Furthermore, in more than 90% of $Ca_V2.1$-knockout PCs, their basal dendrites and somata are innervated by CFs of different neuronal origins. As a result, the proximal somatodendritic compartment in $Ca_V2.1$-lacking PCs receives chaotic innervation by numerous PFs and multiple CFs (Figure 5.6, right). Thus, CF activities leading to AMPA receptor activation and subsequent Ca^{2+} influx through P/Q-type Ca^{2+} channels are essential for monopolizing the proximal dendritic compartment by a single main CF and for expelling other excitatory inputs from that compartment.

Taken altogether, excitatory synaptic wiring in PCs is formed and maintained through homosynaptic competition among CFs and heterosynaptic competition between PFs and CFs. GluRδ2 and Cbln1 fuel heterosynaptic competition in favor of PF innervation, whereas P/Q-type VDCCs facilitate both heterosynaptic and homosynaptic competitions in favor of single main CFs. Based on these molecular mechanisms, PCs establish territorial innervations by PF and CF and mono-innervation by CF.

5.4 DEVELOPMENT OF INHIBITORY SYNAPSES FROM STELLATE CELLS AND BASKET CELLS TO PCS

5.4.1 Formation of Basket Cell–PC Synapses

Basket cells innervate the AIS of PC and constract characteristic pinceau formation in the mature cerebellum (Ito, 1984; Palay and Chan-Palay, 1974). The innervation by basket cell axons of PCs seems to begin when basket cells migrate across the PC layer at the end of the first postnatal week (Ango et al., 2004). The basket cell axons initially form synaptic contacts on the somata of PCs. Then, they appear to move directly to AIS without searching for other possible targets. Upon reaching the AIS, basket cell axons extend multiple terminal branches and eatablish pinceau formation. This behavior strongly suggests that there are guidance cues for basket cell axons along the surface of the PC with gradient from soma to AIS.

Several molecules have been identified that accumulate at AISs of PCs. These include membrane-associated adaptor protein ankyrin-G and one of its binding partner, neurofascin 186 (NF186), a splice variant of neurofascin which belongs to the L1 subgroup of the Ig superfamily (Brummendorf et al., 1998). It is thought that ankyrin-G is stabilized at the AIS partly through its interaction with β4-spectrin tetramers which bind to actin network (Davis et al., 1996). Ankyrins and β-spectrins are known as intracellular adaptor proteins that recruit ion channels, transporters, and cell adhesion molecules to subcellular domains. They are thought to constitute microdomains for intercellular contact and signaling (Bennett and Baines, 2001; Bennett and Chen, 2001). On the other hand, neurofascin is known as a cell-surface glycoprotein that is shown to mediate axon–axon interactions *in vitro* (Rathjen et al., 1987). The distribution of NF186 on PC surface is particularly intriguing. NF186 exhibits subcellular concentration gradient in PCs from AIS toward the soma and dendrites, being highest at AIS and very low at the top of the soma and in the dendrites (Figure 5.7(a); Ango et al., 2004). This gradient is already formed at the end of the first postnatal week when the basket cell axons first contact the somata of PCs. Therefore, it is highly likely that the gradient of NF186 plays an important role in guiding basket cell axons to AIS (Figure 5.7(a)). Since ankyrin-G is expressed exclusively at AISs in PCs, there may be two forms of NF186, the ankyrin-G bound form that is restricted to AIS and the ankyrin-G free form that is distributed to the surface of the PC soma.

Evidence for the requirement of ankyrin-G and NF186 in basket cell axon targeting and pinceau synapse formation at AIS of PC has been presented by the analysis of ankyrin-G knockout mice in which the NF186 gradient in PCs is abolished (Figure 5.7(b); Ango et al., 2004). In these mice, basket cell axons are not restricted to AIS but instead are present on the soma and slightly more distal portion of PC axons (Figure 5.7(b)). Although some basket cell axon bundles successfully reach the AIS, they are very thin, extend along PC axons abnormally, and followed the ectopic localization of NF186. The synaptic contacts visualized by a GABA synthesizing enzyme, GAD65, were greatly reduced at pinceau synapses. These results strongly suggest that NF186 is a substrate for the growth of basket cell axons and its gradient functions as a guidance cue. The results

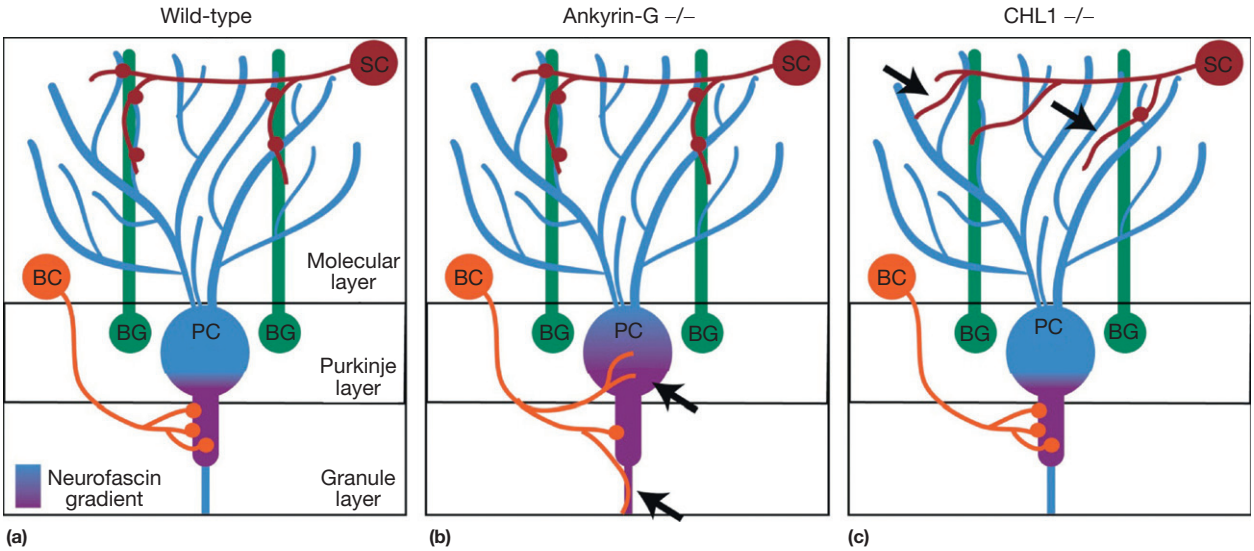

FIGURE 5.7 Subcellular specificity of basket cell and stellate cell inhibitory connections to PCs. Schematics showing the inhibitory synaptic connectivity patterns of the wild-type (a), ankyrin-G-deficient (b), and CHL1-deficient (c) mice. In wild-type mice, a sharp gradient of neurofascin is present from AIS toward the soma. In ankyrin-G-deficient mice, this gradient is no longer restricted to the AIS, which causes mistargeting of basket cell axons and reduced synapse formation. In CHL1-deficient mice, stellate cell axons are not properly guided by Bergmann glia fibers, and synapse formation is decreased. SC, stellate cell; BC, basket cell; PC, Purkinje cell; BG, Bergmann glia. *Reproduced from Williams ME, de Wit J, and Ghosh A (2010) Molecular mechanisms of synaptic specificity in developing neural circuits.* Neuron *68: 9–18, with permission.*

also suggest that NF186 bound to ankyrin-G at AIS is required for the stabilization of pinceau synapses. Further supporting evidence of this notion has been obtained from the experiments to disrupt NF186 function directly in PCs (Ango et al., 2004). Expression of the dominant-negative form of NF186 into PCs during the process of establishing pinceau does not affect AIS-restricted ankyrin-G distribution or guidance of basket cell axons to AIS. However, GAD65-labeled pinceau formation is decreased in the infected PCs to the same extent as in ankyrin-G-deficient PCs. Therefore, either remaining endogenous NF186 can guide the basket cell axons to AIS or NF186 is required specifically for pinceau synapse stabilization while other molecules interacting with ankyrin-G guide the basket cell axons to AIS (Huang et al., 2007; Williams et al., 2010).

5.4.2 Formation of Stellate Cell–PC Synapses

Stellate cells are GABAergic inhibitory interneurons whose somata are located in the molecular layer. In the mature cerebellum, their axons innervate dendrites of PCs with ascending and descending collaterals, and with a plexus of finer branches and terminals. Similarly to basket cells, stellate cells are derived from dividing progenitors in the white matter of postnatal cerebellum (Zhang and Goldman, 1996). The stellate cell precursors migrate into the molecular layer a few days later than the basket cell precursors, with a peak between P8 and P11 but continuing till P14 (Yamanaka et al., 2004). By using a green fluorescent protein- bacterial artificial chromosome transgenic reporter mouse line in which GFP was mainly expressed in stellate cells and basket cells, Huang and colleagues demonstrated how stellate cell axons establish their innervations of PC dendrites after they reach the molecular layer (Ango et al., 2008). Between P12 and P16, stellate cells become bipolar and extend neuritis in horizontal orientation. Then, at P16–P18, stellate cell axons send ascending and descending collaterals, which are further elaborated with appearance of plexus of finer branches up to P40. Importantly, both ascending and descending collaterals of stellate cell axons are strictly associated with the fibers of Bergmann glia that are visualized by the staining of the glia-specific cytoskeleton protein glial fibrillary acidic protein (GFAP). During the third and fourth postnatal weeks, Bergmann glia fibers are known to extend lateral varicoses and fine processes and form an extensive reticular meshwork. Radial fibers from neighboring Bergmann glia are aligned to form palisades in parlobular plane, which are perpendicular to PC dendrites (Altman and Bayer, 1997). In contrast to the close association to Bergmann glia fibers, stellate cell axons do not follow PC dendrites. Bergmann glia fibers enwrap segments of PC dendrites in a patchy, en passant pattern, which is in contrast to the close association to stellate cell axons. Triple immunolabeling of stellate cell axon terminals, Bergmann glia fibers and PC dendrites indicates that stellate cell boutons are formed at the intersection between Bergmann glia fibers and PC dendrites. Thus, Bergmann glia fibers may function as an intermediate scaffold to guide

stellate cell axons along the characteristic trajectories toward multiple PC dendrites and to form synaptic contacts (Figure 5.7(a); Ango et al., 2008).

Since neurofascin, a member of the L1 cell adhesion molecule (L1CAM) subfamily, is crucial for targeting of basket cell axons to the AIS of PC, it is possible that other members of L1CAM might be important for the targeting of stellate cell axons to PC dendrites. A systematic survey of the expression patterns of L1CAMs during postnatal cerebellar development revealed that close homologue of L1 (CHL1) is distributed in a radial stripe pattern that exactly matches the expression of the Bergmann glia marker GFAP, but not the PC marker calbindin (Ango et al., 2008). CHL1 expression in Bergmann glia fibers is prominent as early as P8, reaching higher levels around P18, and then declining in adulthood. CHL1 is also expressed in stellate cells, but the expression is delayed, being undetectable at P8, becoming obvious around P14, and remaining in adulthood (Ango et al., 2008). These expression patterns suggest the involvement of CHL1 in the stellate cell axon targeting to PC dendrites.

A series of experiments using CHL1-deficient mice have clarified the importance of Bergmann glia fibers and CHL1 in organizing stellate cell innervations of PC dendrites (Figure 5.7(c); Ango et al., 2008). In CHL1 knockout mice, stellate cell axons exhibit abnormal trajectories and orientation, and aberrant innervations of PC dendrites (Figure 5.7(c)). In addition, there is a clear reduction in the staining of GAD65, a marker for GABAergic inhibitory synaptic terminal, and the density of stellate cell synapses along PC dendrites. Such aberrant stellate cell axons can form morphologically normal synapses onto PC dendrites albeit with reduced efficacy and density, but many of them are not maintained and the stellate cell axon terminals become atrophic with age. In contrast, there is no change in the GAD65 staining in the AIS of PC (Figure 5.7(c)). Furthermore, there is no change in excitatory synapses from PFs and CFs in CHL1-deficient mice. Importantly, the selective defect in stellate cell innervations and synapse formation on PC dendrites is observed in mice with conditional deletion of CHL1 in Bergmann glia. These results demonstrate that Bergmann glia fibers function as guiding scaffolds, and CHL1 is a molecular signal for organization of stellate cell axon arbors and directing their innervations of PC dendrites (Huang et al., 2007; Williams et al., 2010).

5.4.3 Activity-Dependent Remodeling of Inhibitory Synapses

Several lines of evidence indicate that $GABA_A$ receptor-mediated signaling coordinates pre- and postsynaptic maturation during activity-dependent development of inhibitory synapses (Huang, 2009; Huang and Scheiffele, 2008; Huang et al., 2007). For example, altering GABA synthesis by manipulating the expression of GAD67 greatly influences inhibitory synaptic innervation in the visual cortex (Chattopadhyaya et al., 2007). Acute suppression of the γ2 subunit of $GABA_A$ receptor not only disrupts $GABA_A$ receptor clustering but also reduces innervations of the γ2-deficient neurons by GABAergic terminals (Li et al., 2005). In cerebellar PCs, genetic deletion of the α1 subunit of $GABA_A$ receptor in mice causes complete loss of functional $GABA_A$ receptors and synaptic inhibition in PCs by P18 (Fritschy et al., 2006). Morphologically, GABAergic synaptic terminals from stellate cells are reduced by 75%, whereas basket cell synapses on PC soma are not affected. During postnatal development, GABAergic terminals from stellate cells are initially formed normally onto PC dendritic shafts. PCs of the α1 knockout mice transiently express α3 subunit and have functional $GABA_A$ receptors during early postnatal development (Patrizi et al., 2008). However, subsequent down-regulation of α3 results in complete loss of GABAergic currents and a decreased rate of GABAergic synaptogenesis (Patrizi et al., 2008). Simultaneously, ectopic mismatched synapses begin to be formed between GABAergic terminals and PC dendritic spines (Fritschy et al., 2006; Patrizi et al., 2008) on which normally glutamatergic excitatory terminals make synaptic contacts. Interestingly, the postsynaptic adhesion molecule neuroligin-2 is correctly targeted to inhibitory synapses lacking $GABA_A$ receptors, whereas neuroligin-2 is absent from the mismatched synapses albeit the presence of GABAergic terminals (Patrizi et al., 2008). These results indicate that $GABA_A$ receptors are not required for the formation of synapses, but they appear to be crucial for activity-dependent regulation of synaptic density, presumably through promoting the stabilization of transient axodendritic contact into mature inhibitory synapses.

5.5 SUMMARY AND CONCLUSIONS

The cerebellum provides a good system to study how microcircuits are formed during peri- and postnatal development. The cerebellar cortex consists of only seven types of neurons, that is, PC, GC, basket cell, stellate cell, Golgi cell, Lugaro cell, and unipolar brush cell, and there are two glutamatergic excitatory afferents. The PC is the sole output neuron of the cerebellar cortex and inhibits neurons in the DCN. Bergmann glia, the characteristic astrocyte in the cerebellar cortex, plays multiple roles in neural circuit formation and synaptic transmission such as migration of GCs and guidance of stellate cell axons. The neural circuits made by these cell types and afferents are basically the same throughout the cerebellum.

In the first section of this chapter, we briefly described the cell types and the synaptic organization of the cerebellum and how these cells are generated and migrate to their final positions. We also mentioned the mediolateral compartmentalization based on olivocerebellar projection and some molecular background of the compartmentalization.

In the second section, we made an overview of postnatal development of CF–PC synapses, which is one of the best-studied examples of synapse elimination in the brain. Shortly after birth, each PC is innervated by multiple CFs with similar synaptic strengths on the soma. Subsequently, a single CF is selectively strengthened during the first postnatal week. Then, at around P9, only the strongest CF ('winner' CF) starts to extend its innervation to PC dendrites. In contrast, synapses of the weaker CFs ('loser' CFs) remain on the soma and the most proximal portion of the dendrite, and they are eliminated progressively during the second and third postnatal weeks. From P6 to P11, the elimination proceeds independently of PF–PC synapse formation. From P12 and thereafter, the elimination of weaker CFs requires normal PF–PC synapse formation and is dependent on the PF synaptic inputs that activate mGluR1 and its downstream signaling in PCs.

In the third section, we described how PF synapses are formed and maintained on dendritic spines of PCs. We introduced a recent hot topic that Cbln1 interacts with GluRδ2 on PC dendritic spines and neurexin on PF terminals and that the GluRδ2–Cbln1–neurexin system stabilizes and maintains PF–PC synapses. We also mentioned that mGluR1-mediated calcium signaling in PC dendrites causes release of BDNF and maintains presynaptic function of PFs. Furthermore, we showed that innervation territories of PFs and CFs on PC dendrites stand on the equilibrium caused by heterosynaptic competition between PFs and CFs and homosynaptic competition between multiple CFs.

In the fourth section, we summarized how GABAergic inhibitory synapses from basket and stellate cells are targeted to the PC's AIS and dendrites, respectively. Basket cell axons seem to be guided to PC's AIS following the gradient of neurofascin, a member of L1CAM. This gradient is caused by cross-linking of neurofascin to ankyrin-G that is localized to AISs. On the other hand, stellate cells direct their axons along Bergmann glia fibers to PC dendrites. The association of stellate cell axons to Bergmann glia fibers is mediated by CHL1, another member of L1CAM. We also mentioned that $GABA_A$ receptors appear to be crucial for activity-dependent regulation of the density of inhibitory synapses on PCs.

The cerebellum has been attracting many neuroscientists who pursue the mechanisms of synapse formation, synapse elimination, and synapse remodeling. The small number of cell types and little regional variation in the layer structure and synaptic organization in the cerebellum enable us to perform quantitative and detailed morphological and electrophysiological analyses, when compared to other brain areas. An example is the study of developmental synapse elimination. While this process is intensively studied in peripheral synapses such as neuromuscular junction and autonomic ganglia, it is generally very difficult to do detailed analyses of synapse elimination in the CNS because of small synapse size, heterogeneity and abundance of synaptic inputs to each neuron, and the complexity of synaptic organization. The CF to PC synapse is one of a few examples in the CNS in which developmental synapse elimination can be studied quantitatively by electrophysiological and morphological techniques. Synapses from retinal ganglion cells to the lateral geniculate nucleus and those from the lateral lemniscus to the ventral basal thalamus are also known to undergo massive elimination during postnatal development and have been studied intensively. Formation of inhibitory topographic map in the auditory brainstem is another example in which developmental refinement occurs through synapse elimination. As for the molecular and cellular mechanisms, developmental synapse elimination at CF to PC connection is best characterized among the four types of synapses, as detailed in this review. Thus, CF synapse elimination is an excellent model system for the study of developmental synapse refinement, which is comparable to that of the visual cortex.

The topics introduced in this chapter, that is, CF synapse elimination, maintenance of PF synapses through GluD2-cbln1-neurexin interaction, and targeting of basket cell and stellate cell axons to PCs, are breakthroughs in this field of neuroscience. Continuing researches on cerebellar microcircuits will elucidate fundamental mechanisms of the formation, elimination, maturation, and maintenance of neural circuits in developing CNS.

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