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SYMPOSIUM REVIEW

Calcium-dependent regulation of climbing fibre synapse elimination during postnatal cerebellar development

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Abstract Functional neural circuit formation during postnatal development involves massive elimination of early-formed redundant synapses and strengthening of necessary synaptic connections. In the cerebellum, one-to-one connection from a climbing fibre (CF) to a Purkinje cell (PC) is established through four distinct phases: (1) strengthening of a single CF among multiple CFs in each PC at postnatal age P3–P7 days, (2) translocation of a single strengthened CF to PC dendrites from around P9, (3) early-phase (P7 to around P11) and (4) late-phase (around P12–P17) elimination of weak CF synapses from PC somata. Mice with PC-selective deletion of the P/Q-type voltage-dependent Ca^{2+} channel (VDCC) exhibit severe defects in strengthening of single CFs, dendritic translocation of single CFs and CF elimination from P7. In contrast, mice with a mutation of a single allele for the GABA synthesizing enzyme GAD67 show selective impairment of CF elimination from P10. Electrophysiological and Ca^{2+} imaging data suggest that $GABA_A$ receptor-mediated inhibition onto PC somata from putative basket cells influences CF-induced Ca^{2+} transients and regulates elimination of redundant CF synapses from PC somata at P10–P16. Thus, regulation of Ca^{2+} influx to PCs through VDCCs is crucial for the four phases of CF synapse elimination during postnatal development.

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Introduction

Precise formation of neural circuits during development is a prerequisite for proper functions of the CNS. Neuronal connections are initially redundant, but they are refined and become functionally mature during postnatal development. 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 versions. (Purves & Lichtman, 1980; Katz & Shatz, 1996; Lichtman & Colman, 2000). The cerebellar climbing fibre (CF)–Purkinje cell (PC) synapse provides an excellent model to study synapse elimination in the CNS. While most PCs in the cerebellum of adult mice receive strong excitatory inputs from single CFs (Ito, 1984), PCs in neonatal cerebellum are innervated by multiple CFs with similar synaptic strengths (Hashimoto & Kano, 2003, 2005; Bosman *et al.* 2008; Kano & Hashimoto, 2009). From postnatal day 3 (P3) to P7, one CF is selectively strengthened relative to others in each PC (functional differentiation) (Hashimoto & Kano, 2003, 2005; Bosman *et al.* 2008; Kano & Hashimoto, 2009). Then, only the strengthened CF extends its innervation along growing PC dendrites (CF translocation) (Hashimoto *et al.* 2009). In parallel, surplus CF synapses on the PC soma are eliminated via two distinct mechanisms from P7 to P11 (early phase of CF elimination) and P12 to P16 (late phase of CF elimination). (Hashimoto & Kano, 2003, 2005; Hashimoto *et al.* 2009; Kano & Hashimoto, 2009; Watanabe & Kano, 2011).

Previous studies indicate that CF synapse elimination critically depends on neuronal activity. Decreasing PC activity in mice by PC-selective overexpression of chloride channels impairs CF synapse elimination (Lorenzetto *et al.* 2009). Type 1 metabotropic glutamate receptors (mGluR1) (Kano *et al.* 1997; Ichise *et al.* 2000) and NMDA-type glutamate receptors (Rabacchi *et al.* 1992; Kakizawa *et al.* 2000) have also been shown to be crucial for CF synapse elimination. In addition to these molecules responsible for mediating neural activity, we have recently reported that the P/Q-type voltage-dependent Ca^{2+} channel (P/Q-type VDCC) in PCs (Hashimoto *et al.* 2011) and GABAergic inhibition of the PC soma (Nakayama *et al.* 2012) play crucial roles in distinct phases of CF synapse elimination. P/Q-type VDCCs provide a major route of Ca^{2+} entry into PCs, whereas GABAergic inhibition influences the membrane potential of PCs and the opening of VDCCs, and thereby controls Ca^{2+} influx to PCs. In this Symposium Review, we summarize these two recent papers and discuss how Ca^{2+} transients in PCs regulate CF synapse elimination.

Analysis of PC-selective P/Q-type VDCC knockout mice

Our previous study demonstrates that global P/Q-type VDCC knockout mice have severe defects in CF synapse development including impaired CF synapse elimination and dendritic translocation of multiple CFs (Miyazaki *et al.* 2004). However, it remains unclear to what extent the postsynaptic P/Q-type VDCC in PCs is responsible for these developmental defects and to which phase(s) of CF-PC development the P/Q-type VDCC contributes. To address these issues, we generated mice with a PC-selective deletion of $Ca_V2.1$ (PC-$Ca_V2.1$ KO mice) (Hashimoto *et al.* 2011). The lack of $Ca_V2.1$ mRNA expression was confirmed in PCs at P2. We made whole-cell recordings from PCs in cerebellar slices at P5–P6 and found that voltage-dependent Ca^{2+} currents from PCs were significantly smaller than those of control PCs and were insensitive to the P/Q-type VDCC blocker ω-agatoxin IVA (Hashimoto *et al.* 2011). In contrast, the P/Q channel contributed to the same extent to CF to PC synaptic transmission in PC-$Ca_v2.1$ KO and control mice (Hashimoto *et al.* 2011). These results demonstrate that the P/Q channel at the presynaptic terminal is intact and functions normally in PC-$Ca_v2.1$ KO mice. Then, we performed simultaneous whole-cell recordings and two-photon Ca^{2+} imaging from PCs *in vivo* in control and PC-$Ca_v2.1$ KO mice at P7–P11 under isoflurane anaesthesia (Kitamura *et al.* 2008). We found that PCs exhibited irregular burst firings and occasional trains of burst firing due to CF activity in both PC-$Ca_v2.1$ KO and wild-type mice. However, Ca^{2+} transients induced by spontaneous CF inputs were markedly reduced in PCs of PC-$Ca_v2.1$ KO mice (Hashimoto *et al.* 2011). These results indicate that lack of P/Q-type VDCC in PCs results in reduction of Ca^{2+} influx into PCs at P7–P11 in PC-$Ca_v2.1$ KO mice.

We then examined CF innervations of PCs in PC-$Ca_v2.1$ KO mice and their wild-type littermates. We made whole-cell recordings from PCs in cerebellar slices prepared from control and PC-$Ca_v2.1$ KO mice at various ages, stimulated CFs in the granule cell layer, and examined CF-EPSCs. At P4–P6, no significant difference was observed in the mean number of CFs innervating each PC between PC-$Ca_V2.1$ KO and control mice (Fig. 1*A*). PCs were innervated by more than four CFs in both mice (Fig. 1*A*). Thereafter, PC-$Ca_V2.1$ KO mice manifested severe defects in CF synapse development and elimination (Hashimoto *et al.* 2011). First, CF synapse elimination was severely impaired in PC-$Ca_V2.1$ KO mice until around P12 (Fig. 1). Second, preferential strengthening of single CF inputs from P5 to P7 was severely impaired in PC-$Ca_V2.1$ KO mice (Fig. 2*B* and *C*), despite a comparable 4-fold increase in summed amplitudes of multiple CF-EPSCs from P5 to P8 (Fig. 2*A*). Third, more than one CF translocated to PC dendrites in PC-$Ca_V2.1$ KO mice (Hashimoto *et al.* 2011). These results indicate that Ca^{2+} influx through P/Q-type VDCCs into PCs is crucial for selective strengthening of single CFs, early-phase elimination and selective translocation of single strengthened CFs to PC dendrites.

In addition to severe defects in the postnatal development of CF–PC synapses, we found that PC-$Ca_V2.1$ KO mice exhibit abnormalities in the formation of synapses from parallel fibres (PFs), the other excitatory inputs to PCs. In adult wild-type mice, each PC is innervated by a single CF in proximal dendrites and more than 10^5 PFs in distal dendrites (Ito, 1984; Altman & Bayer, 1997). We found that the extent of CF territory was restricted to the soma and basal dendrites of PCs and PF territory was expanded reciprocally to the proximal somato-dendritic domain in PC-$Ca_V2.1$ KO mice (Miyazaki *et al.* 2012). Consequently, the soma and proximal dendrites of PCs displayed hyperspiny transformation and are innervated by multiple CFs and numerous PFs (Miyazaki *et al.* 2012). These results indicate that Ca^{2+} influx through P/Q-type VDCCs into PCs is crucial not only for homosynaptic competition among multiple CFs but also for heterosynaptic competition between CFs and PFs during postnatal development (Watanabe & Kano, 2011). The schema shown in Fig. 3 illustrates how Ca^{2+} influx through P/Q-type VDCCs regulates multiple events in CF synapse development during the first 10 postnatal days in rodents. CF inputs elicit large AMPA receptor-mediated EPSPs that are large enough to open P/Q-type VDCCs and induce massive Ca^{2+} influx into PC somata. Elevated Ca^{2+} will lead to

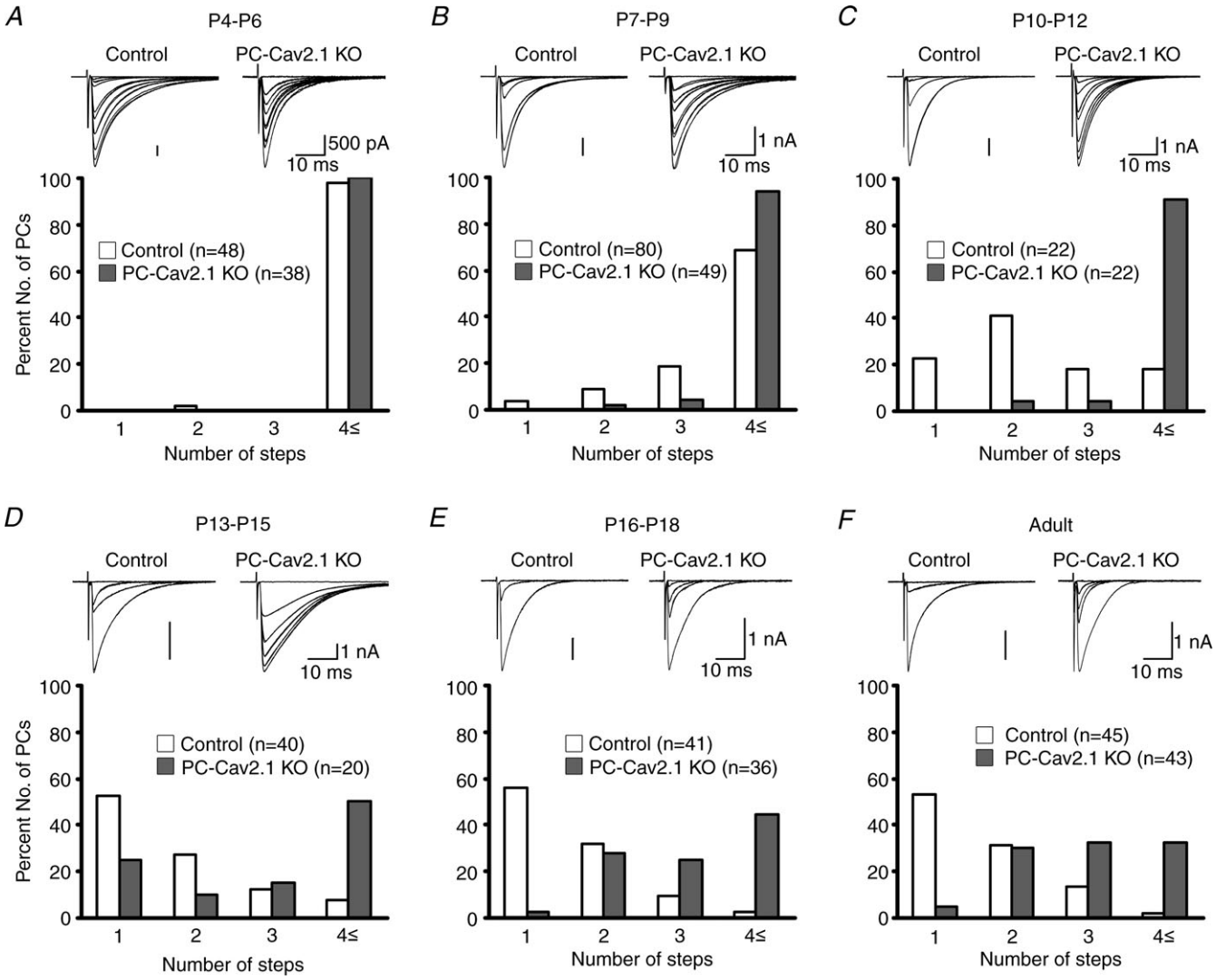

Figure 1. Impairment of CF synapse elimination during postnatal development in PC-$Ca_v2.1$ KO mice
A–F, specimen records of CF-EPSCs (upper panel; two to three traces were superimposed at each threshold intensity; $V_h = -10$ mV) and frequency distribution histogram for the number of discrete CF-EPSCs (lower panel) for control and PC-$Ca_V2.1$ KO mice at indicated ages. There is no significant difference in the frequency distribution in *A* between control and PC-$Ca_V2.1$ KO mice ($P = 0.871$; Mann–Whitney *U* test). In contrast, frequency distributions for *B–F* are significantly different between control and PC-$Ca_V2.1$ KO mice (*B*, $P = 0.023$; *C–F*, $P < 0.001$). Modified with permission from Hashimoto *et al.* (2011).

assignment of 'resources' for synaptic strengthening of respective CF synapses in an activity-dependent manner. This will promote 'functional differentiation' into a single strong CF and several weak CFs in each PC, and will further facilitate 'homosynaptic competition' among multiple CFs. Then, only the strongest CF can start to translocate along growing PC dendrites, which then initiates 'heterosynaptic competition' with PF inputs that begin to expand massively during the second and third postnatal weeks (Fig. 3).

Analysis of GAD67$^{+/GFP}$ mice

To examine whether GABAergic inhibition plays any role in CF synapse development, we used the heterozygous GAD67-GFP (Δneo) knock-in (GAD67$^{+/GFP}$) mouse, which has a deletion of a single allele of a GABA-synthesizing enzyme, GAD67 (Tamamaki *et al.* 2003), as a mouse model of diminished GABAergic transmission. We found that GABAergic transmission was attenuated in GAD67$^{+/GFP}$ mice during the second postnatal week, and that GABA caused inhibition of PC activity after P10 in both control and GAD67$^{+/GFP}$ mice (Nakayama *et al.* 2012). Foliation and the layer structure of the cerebellum, the morphology of PCs, the morphology and density of PF–PC synapses, and basic electrophysiological properties of PF-mediated EPSCs (PF-EPSCs) were normal in GAD67$^{+/GFP}$ mice (Nakayama *et al.* 2012).

In contrast to the normal morphology and function of PF–PC synapses in adult GAD67$^{+/GFP}$ mice (P21–P52),

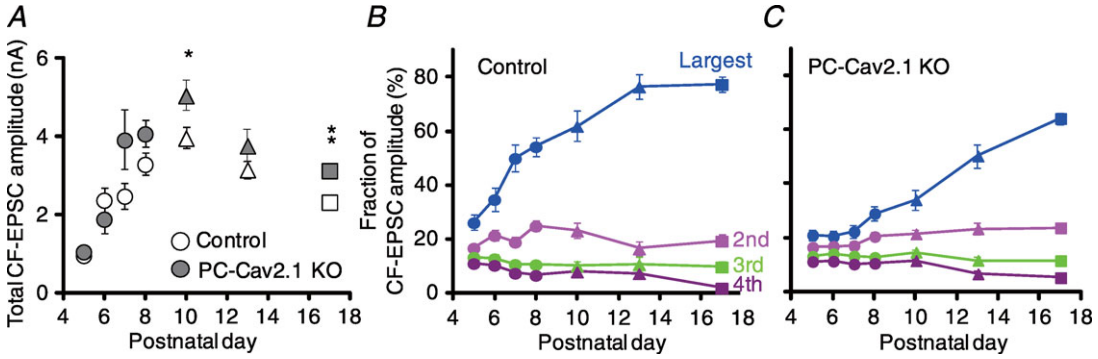

Figure 2. Impairment of postnatal development of CF-PC synapses in PC-Ca$_v$2.1 KO mice
A, postnatal changes in the total amplitudes of CF-EPSCs (at $V_h = -20$ mV) elicited in each PC. Plots include the data for mono-innervating CFs. The numbers of PCs for each data point are 14–77 for control and 10–61 for PC-Ca$_v$2.1 KO PCs. *B, C*, postnatal changes in the fraction of the largest (blue), second (pink), third (green) and fourth (violet) EPSC amplitude relative to the total CF-EPSC amplitude in control (*B*) and PC-Ca$_v$2.1 KO (*C*) PCs. Modified with permission from Hashimoto *et al.* (2011).

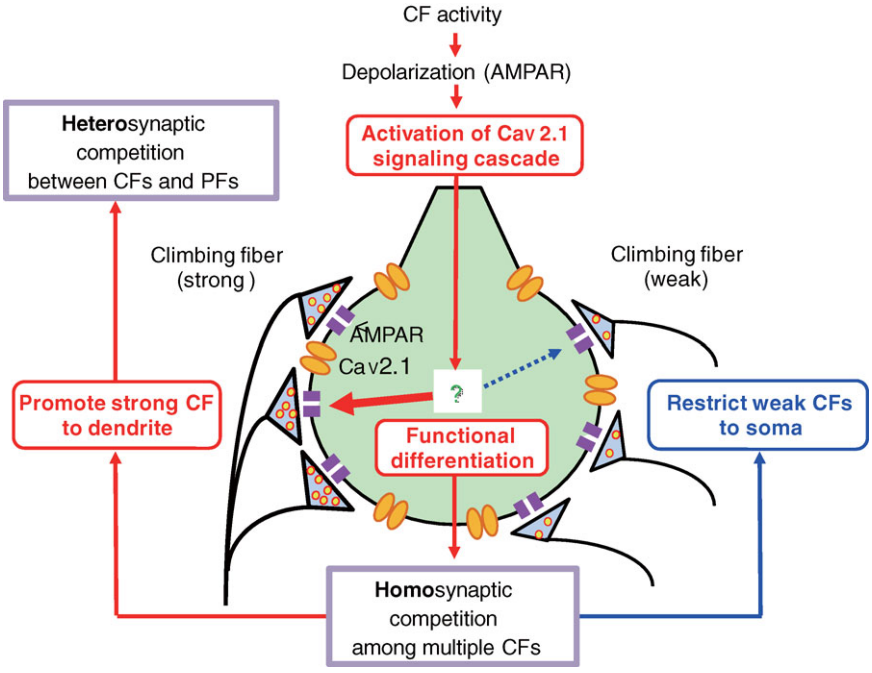

Figure 3. Schema showing how Ca^{2+} influx through P/Q-type VDCCs regulates multiple events of CF synapse development during about the first 10 postnatal days
Modified with permission from Watanabe & Kano (2011).

a higher percentage of PCs were innervated by multiple CFs when compared to control mice (Fig. 4), indicating that developmental CF synapse elimination is impaired in GAD67$^{+/GFP}$ mice. Yet, the basic electrophysiological properties of CF-mediated EPSCs (CF-EPSCs) were normal in GAD67$^{+/GFP}$ mice. During early postnatal development from P5 to P9, there was no significant difference in the mean number of CFs innervating each PC between control and GAD67$^{+/GFP}$ mice (Nakayama *et al.* 2012). Functional differentiation into a single strong CF and several weak CFs in each PC was also normal in GAD67$^{+/GFP}$ mice (Nakayama *et al.* 2012). At P10–P12, the difference between the two genotypes became significant such that control PCs were innervated by significantly fewer CFs than GAD67$^{+/GFP}$ PCs (Nakayama *et al.* 2012). At P13–P15 and P16–P20, the difference became even larger (Nakayama *et al.* 2012). These results indicate that, in GAD67$^{+/GFP}$ mice, initial CF synapse formation, functional differentiation and maturation of CF synapses, and elimination of surplus CFs until P9 are normal, whereas CF synapse elimination after P10 is specifically impaired.

Similar impairment of CF synapse elimination was observed when the GAD inhibitor 3-mercaptopropionic acid (3-MP) was applied locally and persistently to the cerebellum of control mice (Nakayama *et al.* 2012) by means of an ethylene-vinyl acetate copolymer (Elvax) from P10 (Kakizawa *et al.* 2003, 2005). Since 3-MP application from P17 had no effect, the developmental period from P10 to P16 is considered to be the critical period during which CF synapse elimination is sensitive to GAD67 activity in the cerebellar cortex (Nakayama *et al.* 2012). The impairment of CF synapse elimination in GAD67$^{+/GFP}$ mice was reversed when $GABA_A$ receptor sensitivity was enhanced in the cerebellum by local and persistent application of diazepam via Elvax from P10 (Nakayama *et al.* 2012). Again, diazepam application from P17 was ineffective (Nakayama *et al.* 2012), confirming that the GABAergic inhibitory tone from P10 to P16 within the cerebellar cortex is an important factor that regulates developmental CF synapse elimination.

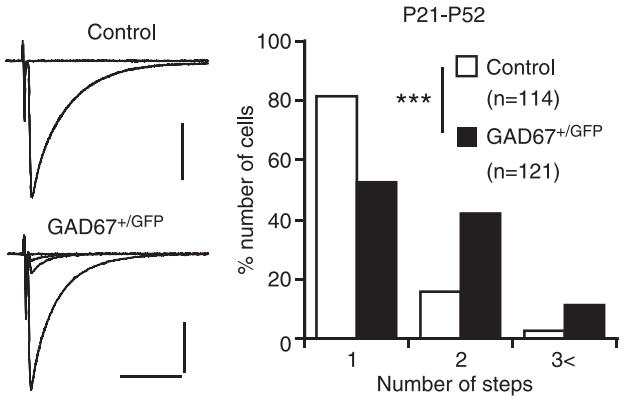

Figure 4. Persistent multiple CF innervation of PCs in mature GAD67$^{+/GFP}$ mice
Left panel, specimen records of CF-EPSCs in a control (P26) and a GAD67$^{+/GFP}$ (P22) PC with gradually increasing stimulus intensities. Two to three traces were superimposed at each threshold intensity. $V_h = -10$ mV. Scale bars, 10 ms and 500 pA. Right panel, summary bar graph showing the number of discrete CF-EPSC steps in PCs from control (□) and GAD67$^{+/GFP}$ (■) mice. Data from 10 control and 11 GAD67$^{+/GFP}$ mice at P20–P52. ***$P < 0.001$. Modified with permission from Nakayama *et al.* (2012).

It has been reported that GABA elicits depolarization and induces Ca^{2+} transients in immature rat PCs (Eilers *et al.* 2001). Therefore, we examined whether GABA excites or inhibits PCs during postnatal development in mice (Nakayama *et al.* 2012). We found that ionophoretic application of the $GABA_A$ receptor agonist muscimol increased the firing rate in about two-thirds of PCs at P4–P6 in both GAD67$^{+/GFP}$ and control mice. By marked contrast, at P10 and thereafter, muscimol decreased the firing rate in most PCs in both strains of mice. This result clearly indicates that GABA inhibits PCs during the developmental period when impairment of CF synapse elimination is manifest in GAD67$^{+/GFP}$ mice (Nakayama *et al.* 2012).

Several types of GABAergic synapses are present in the neural circuitry in the cerebellar cortex and all of them can be affected in GAD67$^{+/GFP}$ mice. We found that miniature IPSCs in PCs with large amplitude (>100 pA, at holding potential of −70 mV) and fast rise time (<1.5 ms) were much less frequent in GAD67$^{+/GFP}$ mice than in control mice (Nakayama *et al.* 2012), suggesting that GABAergic inputs to the PC soma are attenuated in GAD67$^{+/GFP}$ mice. Paired recordings from a putative basket cell (BC) and a PC demonstrated that the amplitude of unitary IPSCs in GAD67$^{+/GFP}$ mice was about half of that of control mice (Nakayama *et al.* 2012), indicating that GABAergic inhibition from putative BCs onto PCs is attenuated in GAD67$^{+/GFP}$ mice.

Reduced GABAergic inhibition is thought to affect depolarization-induced Ca^{2+} transients by CF inputs. We recorded CF-induced EPSPs and Ca^{2+} transients simultaneously from the soma of a PC that was multiply innervated by a single "strong" CF (CF-multi-S) and one or two "weak" CFs (CF-multi-W) (Fig. 5*A* and *B*). Stimulation of such CF-multi-Ws induced Ca^{2+} transients in the PC soma but did not elicit measurable Ca^{2+} elevation in PC dendrites (Fig. 5*B*). Integration of Ca^{2+} transients (for 1.5 s from the onset) in the PC soma by stimulating CF-multi-Ws was significantly larger in GAD67$^{+/GFP}$ mice than in control mice (Fig. 5*B* and *C*). In contrast to CF-multi-Ws, stimulation of CF-multi-S induced large Ca^{2+} transients in PC dendrites, but the magnitudes were not different between control and GAD67$^{+/GFP}$ mice (Fig. 5*B* and *D*). Importantly, bath application of diazepam

(1 μM) significantly reduced the somatic Ca^{2+} transients induced by CF-multi-W stimulation in GAD67$^{+/GFP}$ mice to the same level as those in control mice without diazepam (Fig. 5*E*). Thus, diazepam eliminated the difference in the magnitude of somatic Ca^{2+} transients induced by CF-multi-W stimulation between the two mouse strains, which is considered to be a major cause of diazepam-induced reversal of impaired CF synapse elimination in GAD67$^{+/GFP}$ mice. These results indicate that diminished inhibition of the PC soma permits CF-multi-W to induce much larger somatic Ca^{2+} transients in GAD67$^{+/GFP}$ mice than in control mice. It should be noted that CF-induced Ca^{2+} transients are considered to be mediated mainly by P/Q-type VDCCs (Nakayama

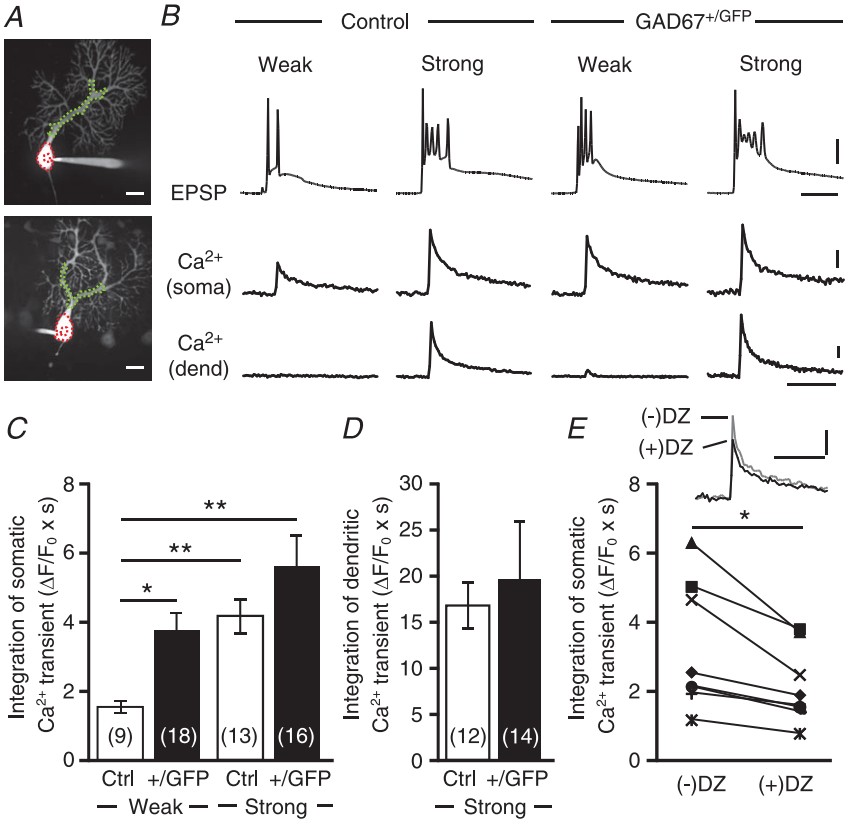

Figure 5. Ca^{2+} transients in the PC soma induced by activation of weak CFs are larger in GAD67$^{+/GFP}$ mice than in control mice
A, representative PC images of a control (upper) and a GAD67$^{+/GFP}$ (lower) mouse. Areas indicated by red and green dotted lines represent ROIs for somatic and dendritic Ca^{2+} transients, respectively. Scale bars, 20 μm. *B*, EPSPs and Ca^{2+} transients recorded in the soma and dendrite of multiply innervated PCs in response to stimulation of a weak or a strong CF in a control and a GAD67$^{+/GFP}$ mouse. Scale bars, 20 mV and 10 ms for EPSPs, 2% (for soma) or 10% (for dendrite) and 1 s for Ca^{2+} transients. *C*, *D*, average magnitudes of Ca^{2+} transients from the PC soma (*C*) and dendrites (*D*) induced by stimulating a weak (CF-multi-W) or a strong (CF-multi-S) CF. Ca^{2+} transients for 1.5 s from the onset were integrated. For the data in *C*, statistical analysis was performed using the Kruskal–Wallis test with the Steel-Dwass multiple comparison *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *E*, traces show somatic Ca^{2+} transients elicited by activation of weak CF in GAD67$^{+/GFP}$ PCs before (–) and after (+) bath application of diazepam (DZ, 1 μM). Data from each cell are plotted in the lower graph. $^{*}P < 0.05$. Scale bars, 2% and 1 s. Modified with permission from Nakayama *et al.* (2012).

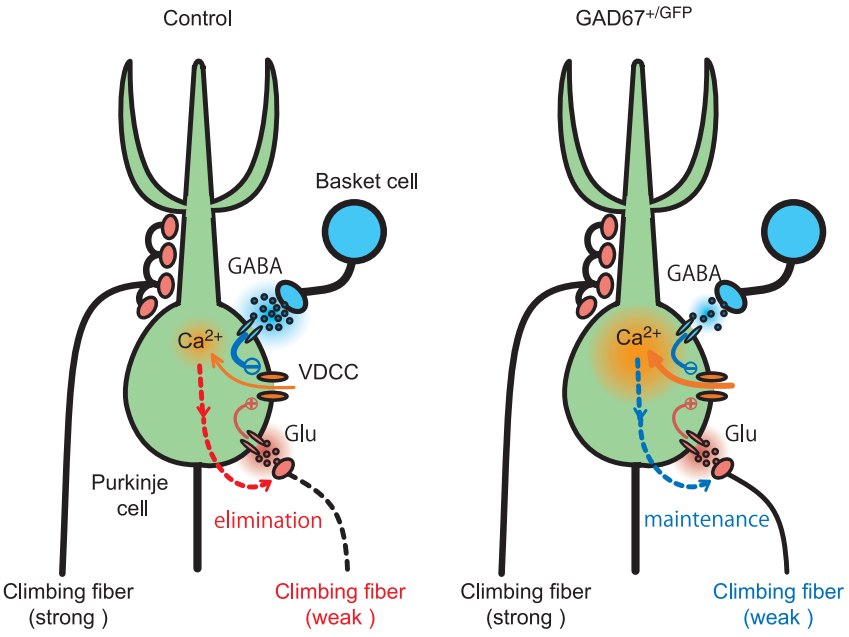

Figure 6. Schema showing a possible mechanism for regulation of the late phase of CF synapse elimination by GABAergic inhibition from BC to PC somata

et al. 2012). Therefore, control of P/Q-type VDCC activity and resultant Ca^{2+} transients by GABAergic inhibition appears to be crucial for CF synapse elimination from P10 to P16.

The schema in Fig. 6 illustrates how GABAergic inhibition to the PC soma regulates the late phase of CF synapse elimination. We hypothesize a mechanism that maintains synapses depending on the Ca^{2+} level of PCs. In wild-type mice, the somatic Ca^{2+} transients induced by CF-multi-W will be smaller than the level of synaptic maintenance because of strong somatic inhibition from putative BCs, and therefore CF-multi-Ws will eventually be eliminated (Fig. 6, left panel). In contrast, the somatic Ca^{2+} transients induced by CF-multi-W in $GAD67^{+/GFP}$ mice will be larger than the level of synaptic maintenance because of the diminished somatic inhibition from putative BCs (Fig. 6, right panel). Consequently, CF-multi-Ws will survive by counteracting developmental synapse elimination, which otherwise prunes CF-multi-Ws during the second postnatal week (Hashimoto *et al.* 2009).

Conclusions

Our analyses of PC-selective P/Q-type VDCC KO mice demonstrate that Ca^{2+} influx through P/Q-type VDCCs into PCs is crucial for strengthening of a single CF among multiple CFs in each PC (functional differentiation), translocation of the single strengthened CF to PC dendrites (dendritic translocation), the early phase of CF elimination, and heterosynaptic competition between CF and PF inputs (Hashimoto *et al.* 2011; Miyazaki *et al.* 2012). In $GAD67^{+/GFP}$ mice, these four events are normal but elimination of redundant CF synapses from P10 to P16, which largely overlaps the period of the late phase of CF elimination, is selectively impaired (Nakayama *et al.* 2012). Our analyses demonstrate that diminished inhibition of the PC soma in $GAD67^{+/GFP}$ mice permits weak CFs to induce Ca^{2+} transients that might be large enough to counteract developmental synapse elimination that otherwise prunes weak CFs during the second postnatal week (Nakayama *et al.* 2012). Since Ca^{2+} transients induced by CF activity are considered to be mediated mostly by P/Q-type VDCCs (Nakayama *et al.* 2012), the results from the analyses of $GAD67^{+/GFP}$ mice indicate that regulation of Ca^{2+} influx through P/Q-type VDCCs to PCs is crucial for the late phase of CF elimination. Thus, activity of P/Q-type VDCCs and resultant Ca^{2+} influx to PCs are key factors in the four phases of CF synapse elimination and heterosynaptic competition between CF and PF inputs. A challenge for future work is to identify the signalling molecules downstream of Ca^{2+} elevation and to elucidate how these molecules contribute to the respective processes of CF synapse elimination and heterosynaptic competition during postnatal cerebellar development.

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