Maintenance of presynaptic function by AMPA receptor-mediated excitatory postsynaptic activity in adult brain

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Activity-dependent synaptic modification occurs in both developing and mature animals. For reliable information transfer and storage, however, once established, synapses must be maintained stably. We investigated how chronic blockade of neuronal activity or α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors affects excitatory climbing fiber (CF) to Purkinje cell (PC) synapses in adult mouse cerebellum. Both treatments caused reduced glutamate concentration transient at the synaptic cleft, decreased frequency of quantal excitatory postsynaptic current, and diminished CF innervation of PC shaft dendrites but no change in CF's release probability. These results indicate that, in the mature cerebellum, AMPA receptor-mediated excitatory postsynaptic activity maintains CF's functional glutamate-release sites and its innervation of PC shaft dendrites.

adult mouse | cerebellar Purkinje cell | climbing fiber | neuronal activity | presynaptic function

Synapses undergo activity-dependent changes not only during development but also in adulthood (1–6). Various forms of synaptic plasticity, including long-term potentiation and depression, have been found in developing and mature animals and have been implicated as cellular bases for learning, memory, and functional neural-circuit formation. Many studies have revealed that neuronal activity is essential for inducing various forms of synaptic plasticity, and underlying mechanisms have been studied intensively (1–6). However, relatively little is known about how the strength of once-established synaptic connection is maintained stably in the mature brain, crucial for reliable information transfer and storage. To examine whether neuronal activity is involved in the maintenance of synaptic strength, it is necessary to detect changes in synaptic responses arising from defined inputs after chronic manipulation of neuronal activity. However, it is difficult to design such experiments, because single neurons in the brain generally receive numerous but weak synaptic inputs from multiple origins.

The climbing fiber (CF)-to-Purkinje cell (PC) synapse in the cerebellum offers a good model system to assess the possible influence of neuronal activity on synaptic strength. In the adult cerebellum, single CFs form hundreds of synaptic contacts onto PC's proximal dendrites and cause strong excitation (7–10). Although PCs receive the other type of excitatory inputs from numerous parallel fibers (PFs), responses elicited by CFs and those by PFs can be easily distinguished electrophysiologically (11, 12). CF activation elicits large and constant excitatory postsynaptic currents (EPSCs) with a low coefficient of variation, making precise and reliable electrophysiological examinations possible (13, 14). Furthermore, amplitudes and kinetics of CF-mediated EPSC (CF-EPSC) are relatively similar when recorded in cerebellar slices prepared from mice of the same strain and age (11, 12, 14–17). We therefore used the CF-to-PC synapse to examine how chronic blockade of neuronal activity or α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

(AMPA)-type glutamate receptors affects synaptic strength in the mature brain. Our results indicate that AMPA receptor-mediated excitatory postsynaptic activity maintains CF's functional release sites and its innervation at PC-shaft dendrites in the mature cerebellum.

Results

Effects of Chronic Tetrodotoxin (TTX) Treatment. To test whether neuronal activity is required for the maintenance of synaptic function in the mature brain, we applied TTX locally to the mouse cerebellum by continuous infusion from ethylene-vinyl acetate copolymer (Elvax) implants (18). At postnatal day (P)24, a small piece of Elvax was implanted on the surface of the cerebellum (lobules 6–8 of the vermis) (15, 16, 19). We estimated the duration of the TTX effect from the performance of Elvax-implanted mice on the rotating rod (15). TTX-treated mice, but not vehicle-treated mice, displayed motor discoordination for about 7 days after Elvax implantation, indicating that TTX blocked activity in the cerebellum during this period (see Fig. 5, which is published as supporting information on the PNAS web site). Therefore, in the following experiments, we performed electrophysiological analyses 3–7 days after the Elvax implantation. We prepared parasagittal cerebellar slices from the vermis and conducted whole-cell recording from visually identified PCs (11, 12, 20, 21). To washout TTX from cerebellar tissues, we incubated slices in a reservoir chamber containing the standard bathing solution for at least 1 h before recording.

In the majority of PCs from the TTX- and vehicle-treated mice, EPSCs were readily elicited in an all-or-none fashion in response to CF stimulation (13, 17), indicating that TTX was sufficiently washed out from cerebellar slices at the time of recording. When PCs were sampled in lobules 6–8 (those closest to the Elvax implant), the average amplitude of CF-EPSCs was significantly smaller in TTX-treated PCs when compared with vehicle-treated PCs (Fig. 1*A* and *B*, $77.3 \pm 3.7\%$, $n = 16$) or untreated control PCs (data not shown). However, when PCs were sampled in lobules 1/2 and 10 (farthest from the implant), no significant difference in the EPSC amplitude was noted between the TTX- and vehicle-treated PCs (Fig. 1*B*). This result indicates that the effect of TTX was confined to the cerebellar lobules near the implants. In the following analyses, we deal with

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Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CF, climbing fiber; Elvax, ethylene-vinyl acetate copolymer; EPSC, excitatory postsynaptic current; CF-EPSC, CF-mediated EPSC; NBQX, 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide; P, postnatal day; PDA, (+)-cis-2,3-piperidine-dicarboxylic acid; PC, Purkinje cell; PF, parallel fiber; PPR, paired-pulse ratio; TTX, tetrodotoxin.

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Fig. 1. Reduced glutamate release from CFs caused by chronic TTX application to the cerebellum. (A and B) Reduced CF-EPSC amplitude after chronic TTX treatment. Specimen traces (A) were obtained from PCs in cerebellar lobules 6–8. Summary bar graphs (B) show that TTX was effective in lobules 6–8 (closest to the implants; $n = 14$ for vehicle, $n = 16$ for TTX) but not in lobules 1/2 and 10 (farthest from the implants; $n = 5$ for vehicle, $n = 5$ for TTX). (C and D) Reduced glutamate-concentration transients at the CF synaptic cleft after chronic TTX treatment. Specimen traces (C) and summary graphs (D) ($n = 6–7$ for vehicle, $n = 4–7$ for TTX) with increasing concentrations of PDA, a rapidly unbinding competitive antagonist of AMPA receptors. (E–G) No change in the amplitude (E and F) but significant reduction in the frequency (E and G) of quantal CF-EPSCs after chronic TTX treatment. Specimen records (E) and cumulative histograms (F and G) were obtained from the same PCs. **, $P < 0.01$; ***, $P < 0.001$; comparison between the TTX- and vehicle-treated samples by Student *t* test.

data obtained from PCs in lobules 6–8. Reversal potential, rise time and decay time constant for CF-EPSC, and PC's membrane capacitance were unchanged by the TTX treatment (see Table 1, which is published as supporting information on the PNAS web site). These results demonstrate that transmission efficacy at CF–PC synapse is weakened by a few days' blockade of neuronal activity.

We tested whether the effect of TTX on CF-EPSCs is reversible. In seven mice, we implanted TTX-containing Elvax and confirmed that TTX was effective by examining the performance of the mice on the rotating rod. Then, we removed Elvax 4 or 7 days after the implantation and performed electrophysiological examination 10–14 days after the removal of Elvax. Amplitudes and other parameters of CF-EPSCs were normal in these mice (data not shown), indicating that the effect of TTX on CF-EPSCs is reversible.

Reduction of transmission efficacy can result from either decreased transmitter release from presynaptic terminals or from reduced receptor sensitivity at the postsynaptic membrane. To assess a possibility of presynaptic change, we asked whether chronic TTX treatment alters glutamate concentration at CF synaptic clefts. We estimated sizes of glutamate transients by analyzing nonequilibrium inhibition of postsynaptic AMPA receptors by (+)-cic-2,3-piperidine-dicarboxylic acid (PDA) (22), a rapidly unbinding competitive antagonist of AMPA receptors. Because PDA is easily displaced by released glutamate (23), its inhibitory effect depends on the amplitude and time course of glutamate in the synaptic cleft. We found that PDA antagonized CF-EPSCs of the TTX-treated PCs more effectively than those of the vehicle-treated PCs (Fig. 1 *C* and *D*). To determine the locus of this difference, we recorded quantal CF-EPSCs in the presence of Sr^{2+} , which causes asynchronous transmitter release (24, 25) (Fig. 1*E*). The mean amplitude of quantal CF-EPSCs and their distribution were unchanged by the TTX treatment ($P = 0.2111$, Kolmogorov–Smirnov test; Fig. 1 *E* and *F*), indicating no change in the quantum size. In contrast, the frequency of quantal CF-EPSCs was significantly ($P = 0.0267$) lower in the TTX-treated PCs (Fig. 1 *E* and *G*). These results strongly suggest that the

reduced glutamate transients in the TTX-treated PCs results from decreased glutamate release from CFs.

Effects of Chronic 2,3-Dioxo-6-Nitro-1,2,3,4-Tetrahydrobenzoquinoline-7-Sulfonamide (NBQX) Treatment. To examine whether excitatory postsynaptic activity is required for the maintenance of CF's presynaptic function, we applied the AMPA-receptor antagonist NBQX locally to the cerebellum by means of Elvax. We estimated that the duration of the NBQX effect was ≈ 7 days after Elvax implantation by the performance of Elvax-implanted mice on the rotating rod (15) (Fig. 5). By performing *in vivo* extracellular recording in the cerebellum of anesthetized mice, we confirmed that NBQX effectively blocked CF-mediated excitation of putative PCs (see *Supporting Text* and Fig. 6, which are published as supporting information on the PNAS web site). We also examined whether NBQX influences glutamate release from CFs in acute slices. CF-EPSCs were elicited by paired CF stimuli at an interval of 100 ms, and paired-pulse ratio (PPR), which is known to be a good measure of release probability (26), was monitored. As detailed in *Supporting Text* and Fig. 7, which is published as supporting information on the PNAS web site, NBQX did not affect PPR, indicating that NBQX has no effect on glutamate release from CFs.

We then examined the effects of chronic NBQX treatment and found that it had essentially the same effects on CF-PC synapses as those exerted by the TTX treatment (Fig. 1). For PCs sampled in cerebellar lobules 6–8 but not in lobules 1/2 and 10, the average CF-EPSC amplitude was significantly smaller in the NBQX-treated than in the vehicle-treated mice (Fig. 2*A* and *B*, $52.1 \pm 4.3\%$, $n = 13$). Basic electrophysiological parameters of PCs were not altered by the NBQX treatment (Table 1). PDA antagonized CF-EPSCs of the NBQX-treated PCs more effectively than those of the vehicle-treated PCs (Fig. 2*C* and *D*). The mean amplitude of quantal CF-EPSCs and their distribution were unchanged ($P = 0.4962$, Kolmogorov–Smirnov test; Fig. 2*E* and *F*), whereas the frequency was significantly ($P = 0.0011$) lower in the NBQX-treated PCs (Fig. 2*E* and *G*). These results clearly indicate that a few days' blockade of postsynaptic AMPA receptors significantly reduces glutamate release from CFs, with no perceptible changes in postsynaptic glutamate sensitivity.

Fig. 2. Reduced glutamate release from CFs caused by chronic NBQX application to the cerebellum. (A and B) Reduced CF-EPSC amplitude after chronic NBQX treatment. Specimen traces from PCs recorded near the Elvax implants (A) and summary bar graphs for CF-EPSC amplitudes (B) in lobules 6–8 ($n = 14$ for vehicle, $n = 13$ for NBQX) and lobules 1/2 and 10 ($n = 5$ for vehicle, $n = 5$ for NBQX). (C and D) Glutamate-concentration transients at synaptic cleft. Sample traces (C) and summary graphs (D) ($n = 6–7$ for vehicle, $n = 7$ for NBQX) with increasing concentrations of PDA. (E–G) No change in the amplitude (E and F) but significant reduction in the frequency (E and G) of quantal CF-EPSCs after chronic NBQX treatment. Specimen records (E) and cumulative histograms (F and G) were obtained from the same PCs. **, $P < 0.01$; ***, $P < 0.001$; comparison between the NBQX- and vehicle-treated samples by Student *t* test.

To check whether TTX and NBQX have additive effects on CF synapses, we tested Elvax containing both TTX and NBQX. We found that the effect of TTX plus NBQX ($54.0 \pm 5.0\%$ of control, $n = 10$) was similar to that of NBQX alone ($52.1 \pm 4.3\%$ of control; $n = 13$) (see Fig. 8, which is published as supporting information on the PNAS web site), indicating that NBQX occludes the effect of TTX. This result suggests that TTX and NBQX exert their actions through common mechanisms.

Chronic TTX or NBQX Treatment Induces Reduction of CF's Functional Release Sites. It has recently been clarified that multivesicular release occurs at CF–PC synapses at normal extracellular Ca^{2+} (14, 27, 28). Decreasing release probability by lowering extracellular Ca^{2+} to 0.5 mM or lower results in one-site-one-vesicle release (27). To elucidate the causes of smaller glutamate transients in the TTX- or NBQX-treated PCs, we estimated glutamate transients resulting from single synaptic vesicles in a low external Ca^{2+} (0.5 mM) saline (14, 27, 28). Blocking effects of PDA were the same for the TTX-, NBQX-, and vehicle-treated PCs at all PDA concentrations tested (Fig. 3 A and B),

indicating that glutamate transients resulting from single synaptic vesicles are the same. Furthermore, blocking effects of PDA in normal (2 mM) and low (0.5 mM) external Ca^{2+} were almost the same for the TTX- (Fig. 3C) and the NBQX-treated (Fig. 3D) PCs. Therefore, the difference in glutamate transients in normal external Ca^{2+} is most likely to result from the lower probability of multivesicular release in the TTX- or NBQX-treated PCs.

Multivesicular release depends critically on both release probability and the number of functional release sites per synapse. To estimate release probability, we examined paired-pulse plasticity (26). In low extracellular Ca^{2+} (0.25 mM and 0.5 mM), in which CF-EPSCs were not saturated (28), PPR was similar for the three conditions of PCs (Fig. 3 E and F). This result indicates that there is no significant difference in CF's release probability in the three conditions of PCs. In normal extracellular Ca^{2+} (2 mM), PPRs for the TTX- and NBQX-treated PCs were significantly smaller than for the vehicle-treated PCs (Fig. 3F). Previous studies show that postsynaptic receptors are saturated in extracellular $\text{Ca}^{2+} > 1$ mM (28). The

Fig. 3. No changes in quantum size and release probability of CFs. (A and B) Glutamate transients were estimated in low external Ca^{2+} (0.5 mM) saline in which one-site-one-vesicle release occurred from each CF terminal. Specimen traces (A) and summary graphs (B) ($n = 7$ for each group) with increasing concentrations of PDA. (C and D) Glutamate transients were estimated in the normal (2.0 mM) and low (0.5 mM) external Ca^{2+} saline for the TTX-treated (C) ($n = 7$) and NBQX (D) ($n = 7$) PCs. *, $P < 0.05$; comparison between data in the normal (2.0 mM) and low (0.5 mM) external Ca^{2+} saline in the TTX-treated PCs (C) by Student *t* test. (E and F) Paired-pulse plasticity tested in 0.25 mM, 0.5 mM, and 2.0 mM external Ca^{2+} ($n = 6$ for vehicle, $n = 5–13$ for TTX, and $n = 7–14$ for NBQX). *, $P < 0.05$; **, $P < 0.01$; comparison between the TTX- or NBQX-treated PCs and the vehicle-treated PCs by Student *t* test.

Discussion

Chronic TTX or NBQX Treatment Induces Functional Weakening of CF Synapses. It has been demonstrated morphologically that chronic infusion of TTX into the rat cerebellum results in retraction of CF innervation territory on PC dendrites (9, 30). We have not only described electrophysiological changes in CF–PC synapses after chronic TTX treatment but also found two important results. First, blockade of postsynaptic AMPA receptors by NBQX caused essentially the same changes as nonspecific blockade of neuronal activity by TTX, suggesting the existence of some retrograde signaling mechanism from postsynaptic neuron to presynaptic terminals. Second, chronic TTX or NBQX treatment induced not only morphological retraction of CF innervation but also clear reduction in the number of functional release sites per active zone at individual CF terminals. Thus, this study has made significant advances over the morphological observation reported in refs. 9 and 30).

AMPA Receptor-Mediated Neuronal Activity Maintains Mature Properties of CF Synapses. We have shown that immature CFs in developing cerebella have lower probability of multivesicular release (14). We show here that the TTX or NBQX treatment in the mature cerebellum induced significant weakening of the CF–PC synaptic strength, with decreased probability of multivesicular release, similar to the properties of immature CFs (14). These results suggest that AMPA receptor-mediated activity is required for the maintenance of mature properties of glutamate release from CFs in the adult cerebellum. NBQX treatment not only reduces PC excitability due to blockade of AMPA receptor-mediated excitatory drive but also may reduce PF activity by silencing granule cells. However, CF-EPSC is not attenuated by reduced PF activity caused by chronic blockade (from P17–P24) of NMDA receptors at mossy-fiber-to-granule-cell synapses (15). Furthermore, in several mouse models, including GluR δ 2-deficient mice (31, 33), impairment of PF–PC-synapse formation and resultant reduction of PF drive of PCs causes expansion of CF territory. Therefore, the retraction of CF territory in the NBQX-treated mice is not likely to result from reduced PF activity.

The NBQX treatment may also block Ca^{2+} -permeable AMPA receptors on Bergmann glia. Alteration of glial AMPA receptors to Ca^{2+} -impermeable ones results in retraction of glial processes and multiple CF innervation of PCs (34). However, the normal morphology and synapse enwrapping of Bergman glia and the lack of multiple CF innervation in the NBQX-treated mice indicate that the changes in CF's presynaptic function was not caused by reduced Ca^{2+} influx to Bergman glia through AMPA receptors. Therefore, we conclude that the changes in the NBQX-treated mice resulted mainly from blockade of excitatory postsynaptic activity in PCs.

Apparent Difference in the Effects of TTX and NBQX. It has been reported that chronic infusion of TTX to the mature rat cerebellum causes significant regression of CF innervation, induces hyperspiny transformation of PC soma and proximal dendrites, and permits proximal expansion of PF-synapse territory by innervating the ectopic spines (9, 30, 35). Although the chronic NBQX application to the mature mouse cerebellum via Elvax caused apparent regression of CF innervation, the application had no perceptible effect on the morphology of PF–PC synapses and spine formation at the somatodendritic domain. We assume that blockade of both pre- and postsynaptic activity by TTX might be required for clear morphological changes in PF–PC synapses and for the hyperspiny transformation.

The effect of chronic NBQX treatment appears to be stronger than that of TTX. We assume that the difference may be partly because of the possible contribution of inhibitory synaptic

inputs. Whereas TTX blocks both excitatory and inhibitory synaptic inputs, NBQX selectively blocks excitatory inputs. If excitatory and inhibitory inputs have opposite effects on the maintenance of CF synapses, the effect of NBQX would be larger than that of TTX.

Possible Mechanisms of AMPA Receptor-Mediated Maintenance of CF Synapses. We have reported that CF-innervation territory is significantly diminished to the proximal portion of PC dendrites in mutant mice deficient in P/Q-type Ca^{2+} channel α 1A subunit (31, 32). The diminished CF innervation of α 1A knockout mice is similar to that observed in NBQX-treated mice, in which CFs often stop innervation in the midst of shaft dendrites, leaving the remaining distal part free of CF innervation. Although there is no direct evidence, the similar phenotype in these two mouse models suggests that the same defect in Ca^{2+} -dependent signaling in PCs may underlie the diminished CF innervation.

The present results, that presynaptic functions are maintained by postsynaptic AMPA receptor-mediated activity, suggest that some transsynaptic retrograde signaling mechanism must exist to inform presynaptic terminals of postsynaptic activity. One possibility would be that some diffusible retrograde messengers are released from PCs and act on CF terminals. There is increasing evidence that PCs release endocannabinoids and/or glutamate which act as retrograde messengers and modulate excitatory synaptic transmission onto PCs (36–39). These molecules may play a role also in the maintenance of CF synapses. Cytokines are other candidate molecules. We have shown that insulin-like growth factor 1 supports CF–PC synapses in the developing cerebellum during P8–P12. The effects are not observed after P12 (15, 16, 19), and, therefore, insulin-like growth factor 1 itself may not be involved in the maintenance of CF synapses in the mature cerebellum. Nevertheless, other cytokines that have similar effects on CF synapses may play a role in the mature cerebellum. Another possibility would be that molecules that bridge pre- and postsynaptic sites may mediate retrograde signals by protein–protein interaction. For example, certain cell-adhesion molecules might be maintained in a manner that depends on the postsynaptic Ca^{2+} level. Blockade of AMPA receptor-mediated postsynaptic activity and the resultant decrease in Ca^{2+} influx might degrade the integrity of such molecules, leading to retraction of CF terminals from PC dendrites.

Although a number of previous studies indicate that activity-dependent refinement of synaptic connection is manifest during postnatal development, such change can occur in mature animals as well. For example, somatotopic maps in the sensory cortex can change dynamically after partial deprivation of sensory inputs or after change in the pattern of sensory inputs (40–42). NMDA receptor-mediated neuronal activity appears to be required for reorganization of somatosensory maps in the adult cortex (43). The present findings that AMPA receptor-mediated postsynaptic activity maintains presynaptic functions have disclosed a new aspect of activity-dependent modification of adult neural circuitry. Further elucidation of the mechanisms of the NMDA receptor-dependent and AMPA receptor-dependent mechanisms will deepen our understanding of how synaptic connections are maintained in the mature nervous system.

Methods

Experiments were conducted according to the guidelines of the Animal Welfare Committee of Kanazawa University. To rule out possible experimenter bias, all experiments have been done blinded to the treatments of the mice.