Impaired Motor Coordination Correlates with Persistent Multiple Climbing Fiber Innervation in PKCγ Mutant Mice

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Summary

It is generally believed that a smooth execution of a compound movement, or motor coordination, requires learning of component movements as well as experience-based refinement of the motor program as a whole. PKCγ mutant mice display impaired motor coordination but intact eyeblink conditioning, a form of component movement learning. Cerebellar long-term depression, a putative cellular mechanism for component motor learning, is also unimpaired. Thus, PKCγ mutant mice are defective in refinement of the motor program. In the accompanying paper, we demonstrate that innervation of multiple climbing fibers onto Purkinje cells persists in adulthood in these mutant mice. We propose that this defective elimination of surplus climbing fibers underlies motor discoordination.

Introduction

The classic neurological concept that the cerebellum is a site of motor control originates from a number of observations that patients with cerebellar damages often exhibit motor-related clinical symptoms such as gait ataxia, dysmetria, hypotonia, and tremor (Dow and Moruzzi, 1958). Thach et al. (1992) suggest that the adaptive role of the cerebellar cortex is to combine simpler elements of movement into more complex coordinated acts. They also suggest that the cerebellum is the apparatus that allows simple, stereotyped reflexes to respond appropriately under different task conditions. These notions imply two distinct functions of the cerebellum: learning associated with component movements (discrete motor learning), and per-

forming compound movements smoothly (motor coordination).

Evidence has been accumulated suggesting that the memory traces for discrete motor learning, such as adaptation of vestibulo-ocular reflex (VOR) and classical conditioning of eyeblink response, are stored in the cerebellum (for VOR, see Ito, 1984; for eyeblink, see McCormick and Thompson, 1984; Yeo et al., 1984; Lavond et al., 1985; Thompson, 1986; Krupa et al., 1993; Daum et al., 1993; Molchan et al., 1994). The synaptic plasticity, long-term depression (LTD) at parallel fiber (PF)–Purkinje cell (PC) synapses, has been suggested to be a cellular substrate for these forms of discrete motor learning (for VOR, see Ito et al., 1982; Ito, 1989, 1994; for eyeblink, see Thompson et al., 1993; Chen and Thompson, 1995). Furthermore, LTD has been proposed to underlie motor coordination (Ito, 1984). However, there has been little direct evidence linking LTD to discrete motor learning or motor coordination.

Gene knockout mice could potentially be useful in assessing the relationship between phenomena observed at different levels of complexity (for reviews, see Grant and Silva, 1994; Mayford et al., 1995; Chen and Tonegawa, 1995). For instance, mice lacking the functional gene for the metabotropic glutamate receptor type 1 (mGluR1) are deficient in the cerebellar LTD and are impaired in eyeblink conditioning (Aiba et al., 1994; Conquet et al., 1994). These data strengthen the notion that the LTD is a cellular substrate for discrete motor learning.

In the present study, we have analyzed the mutant mice deficient in the γ isoform of protein kinase C (PKCγ). PKCγ is brain-specific and is highly expressed in cerebellar PCs (Huang et al., 1988; Kose et al., 1988; Saito et al., 1988; Tanaka and Saito, 1992; for review, see Tanaka and Nishizuka, 1994). The expression of PKCγ reaches a transient peak in the third postnatal week (Hashimoto et al., 1988; Herms et al., 1993; Moriya and Tanaka, 1994), which corresponds temporally to a transition from multiple CF innervation to a single CF innervation to each PC (Crepel, 1982). Furthermore, PKC has been implicated in LTD (Crepel and Krupa, 1988; Crepel and Jaillard, 1991; Linden and Connor, 1991; for reviews, see Linden, 1994; Hemart et al., 1995).

In the accompanying paper in this issue of *Cell* (Kano et al., 1995), we report that about 40% of PCs in mature PKCγ mutant mice remain multiply innervated by CFs. In this paper, we report that these mutant mice are defective in motor coordination but are fully capable of discrete motor learning, and LTD is fully inducible in the cerebellar slices. Comparing these findings on PKCγ mutant mice with the phenotypes of several other gene knockout mice described in recently published work (Aiba et al., 1994; Kashiwabuchi et al., 1995; K. Shibuki et al., submitted), we propose that the primary cause of the observed motor discoordination in PKCγ mutant mice is the persistent multiple innervation of PCs by CFs.
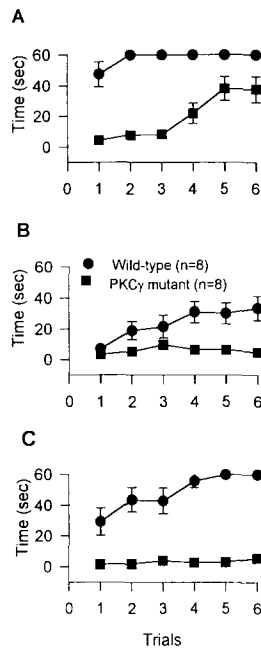
Figure 1. PKCγ Mutant Mice Are Impaired in Motor Coordination

(A) The stationary rotorod. Wild-type mice (n = 8) were able to stay on the roller for an extended period of time (mean ± SEM, 60 s allowed maximally); although they improved considerably after three trials, mutant mice (n = 8) spent significantly less time on the stationary rotorod than did the wild type (F[1,95] = 244.9, P < 0.0001).

(B) Running rotorod (8 PRM). Initially, both wild-type and mutant mice fell off the running roller immediately. Wild-type mice showed a steady improvement over trials, while mutant mice did not improve at all (F[1,95] = 134.5, P < 0.0001).

(C) Horizontal thin rod. Wild-type mice eventually managed to remain on the rod for an extended period of time, but mutant mice failed to do so (F[1,95] = 38.3, P < 0.0001).

Results

Motor Coordination Is Impaired

PKCγ mutant mice are impaired in several motor coordination tasks (Figure 1). For instance, the mutant mice had difficulty in staying on the stationary rotorod, although practice improved the performance (Figure 1A). Nevertheless, even after six trials, the staying duration by the mutant mice was significantly shorter than that of the wild-type mice. When the rotorod was running, wild-type mice showed a steady improvement over trials, but mutant mice did not improve at all (Figure 1B). When placed on a thin rod, wild-type mice eventually managed to remain on the rod for the period of time allowed (60 s), but mutant mice failed to do so (Figure 1C). Thus, although they retain certain limited ability to manage simple coordinated acts such as staying on the stationary rotorod, PKCγ mutant mice could not adapt to more challenging tasks such as staying on the running rotorod or the thin rod.

PKCγ mutant mice showed a mild ataxic gait but not any sign of tremor. A slight wobbling could be detected by close observations, which became more visible during running. Mutant mice typically walk faster than wild-type mice, and they tend to be more active. By examining the footprint pattern during walking, we noticed that mutant mice walked with a shorter pace (Figure 2).
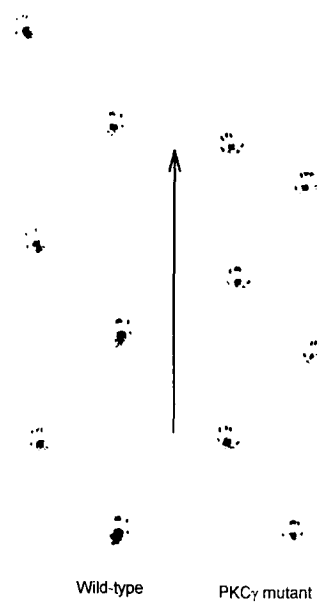

Wild-type   PKCγ mutant

Figure 2. Hind Footprint Pattern during Walking

The PKCγ mutant mouse walks with a shorter pace and slight wobbling. Wobbling becomes more visible when the mutant mouse speeds up (data not shown).

Eyeblink Conditioning Is Facilitated

We investigated simple associative learning of discrete motor responses in PKCγ mutant mice. We found that PKCγ mutant mice showed eyeblink conditioning following pairings of an initially neutral tone with a 100 ms periocular shock (Figure 3). The general features of the conditioned eyeblink response resembled those previously reported for rabbits (Gormezano et al., 1962), rats (Skelton, 1988), and mice (Aiba et al., 1994). The increase of electromyograph (EMG) activity following a presentation of a tone represents a conditioned response (CR), and the activity following the shock is an unconditioned response (UR)
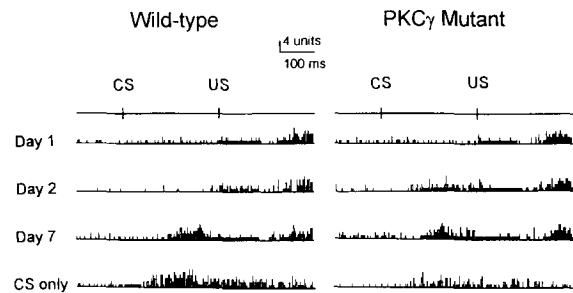

Figure 3. Examples of Discriminated EMG Activity during Associative Eyeblink Conditioning in Wild-Type and Mutant Mice

Examples of eyeblink response to paired tone–shock presentations in the initial (day 1), intermediate (day 2), and well-trained stages (day 7) in wild-type (left) and mutant (right) mice. The bottom traces are conditioned response to tone-alone trials (CS only). Onsets of tone (CS) and shock (US) are indicated in the top of the figure. Discriminated EMG activity elicited by the tone is a CR. Owing to saturation of the amplifier during the US period, the increase in EMG activity upon termination of the shock is recorded as a UR.
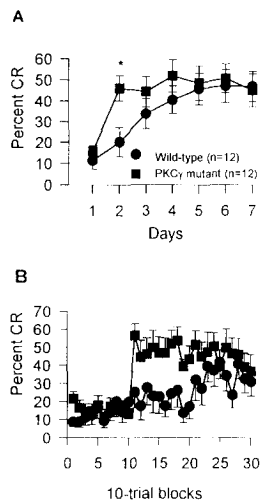
A

B

Figure 4. PKCγ Mutant Mice Show Facilitated Eyeblink Conditioning

(A) Percent CR by session. Wild-type mice (n = 12) showed gradual increase in percent CR (mean ± SEM) over first 4 days of training. In contrast, mutant mice (n = 12) exhibited asymptotic CRs by day 2 of training. Percent CR in mutant mice is significantly higher than wild-type mice on the second day of training, as marked by an asterisk (F[1,23] = 8.29, p < 0.01), while no statistically reliable differences were observed in other days.

(B) Percent CR by block was calculated for each 10-trial block for the first 3 days of training. The facilitation of eyeblink conditioning in mutant mice is most apparent from block 20 to block 30 (corresponding to the second session in [A]).

(Figure 3). We used two indicators for assessing learning, one being percent CR and the other being CR amplitude. CR was scored by statistical comparison between EMG activity triggered by a tone and the pre-tone baseline (see Experimental Procedures for more details).

To our surprise, PKCγ mutant mice showed a facilitated
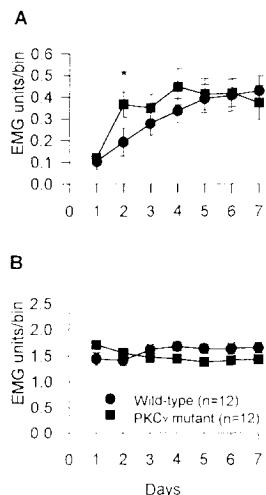
A

B

Figure 5. Amplitudes of CR and UR during Eyeblink Conditioning in Wild-Type and Mutant Mice

(A) CR amplitude. Mutant mice exhibit significantly higher CR amplitudes than wild-type mice on the second day, as marked by an asterisk (F[1,23] = 4.35, p < 0.05), but not on subsequent days.

(B) UR amplitude. On US-alone trials, UR amplitudes, measured immediately following the shock, do not differ between the two groups.
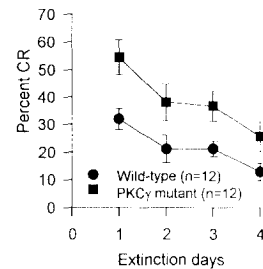

Figure 6. Mutant Mice Show More Memory Saving of Conditioned Responses during Extinction Test

After being switched to CS-alone trials, both groups demonstrate a gradual extinction of percent CR over 4 days. But percent CR is not yet extinguished to preconditioning baselines (see day 1 in Figure 4A). The percent CR in the mutant group is consistently higher than in the wild type (F[1,23] = 9.30, p < 0.01).

learning of CRs, as shown by higher percent CRs exhibited in mutant mice (Figure 4). This facilitation of eyeblink conditioning was most visible on the second day of training. On subsequent days, mutant and wild-type mice reached a similar level of conditioning. The facilitation of CRs in mutant mice was also seen as larger CR amplitudes during the second day of training (Figure 5). As with percent CR, the CR amplitudes of mutant and wild-type mice became similar as the training continued. In contrast, the amplitudes of URs did not change over the course of training and did not differ between wild-type and PKCγ mutant groups (Figure 5). Thus, the facilitation in PKCγ mutant mice seemed to occur specifically for the CR and not for the UR.

The notion that the associative learning by PKCγ mutant mice is facilitated was also supported by the extinction test (Figure 6). When switching to conditioned stimulus (CS)-alone trials, the percent CR in the mutant group was consistently higher than that in the wild-type group during the course of extinction, but the rate of extinction did not appear to be different between the two groups. One possibility is that the facilitated learning by PKCγ mutant mice leads to more memory savings. Although the memory savings are not shown in either percent CR or CR amplitudes during the late phase of training, possibly owing to a ceiling effect of behavioral expression, savings can be seen more clearly during extinction (Figure 6).

LTD Is Present in PF–PC Synapses

To examine LTD of PF–PC transmission, we measured excitatory postsynaptic currents (EPSCs) with a whole-cell recording technique of PCs in cerebellar slices from 16- to 25-day-old mice. PFs were stimulated in the middle molecular layer, about 200 μm away from the recorded PCs. To induce LTD, we used a conjunction protocol that is composed of 240 PF stimuli in conjunction with a depolarizing pulse (200 ms, −60–+0 mV) repeated at 1 Hz.

In the wild-type PCs, the conjunction protocol induced a significant decrease in the amplitude of PF-EPSCs (Figure 7A), which persisted for the full 35 min during which responses were monitored. The magnitudes of LTD measured during a 25–35 min period after conditioning range from 59% to 95% (76.5% ± 4.3%, n = 12 from nine

Normal Intracellular Solution

A wild type
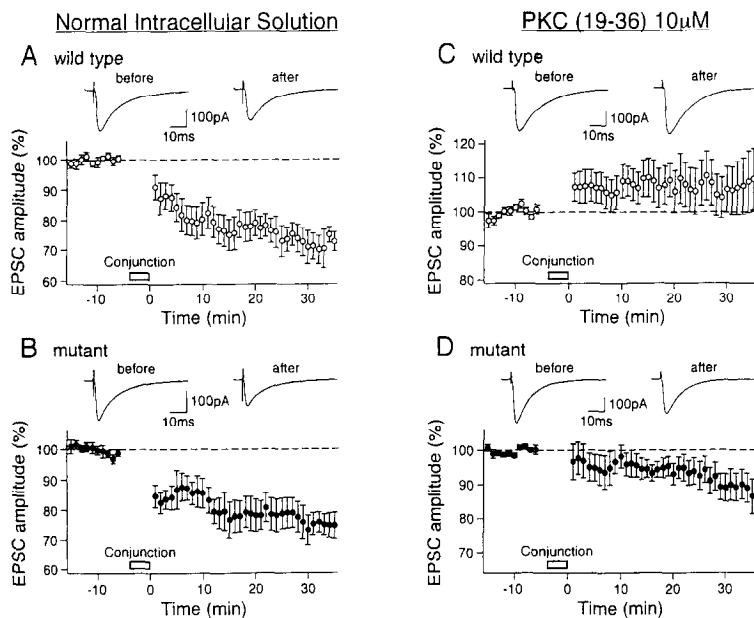

B mutant

PKC (19-36) 10μM

C wild type

D mutant

Figure 7. LTD of PF–PC Synapses Appears Normal, but the Efficacy of a Specific PKC Inhibitor to Block LTD Is Altered in PKCγ Mutant Mice

(A) LTD was induced by a conjunction protocol in wild-type PCs (mean ± SEM, n = 12 from nine mice, P16-P25, five cells studied blind). This protocol comprises 240 PF stimuli in conjunction with a depolarizing pulse (200 ms, −60–+0 mV) repeated at 1 Hz.

(B) LTD was induced by the same conjunction protocol as in (A) in mutant PCs (n = 9 from six mice of P17-P26, six cells studied blind).

(C) LTD was blocked in wild-type PCs (n = 12 from seven mice of P18-P23, three cells studied blind), when the pipette solution contained a specific peptide inhibitor of PKC, PKC(19–36).

(D) LTD was not totally abolished in mutant PCs (n = 11 from six mice of P18-P23, five cells studied blind) with the pipette solution containing PKC(19–36).

Each point represents the amplitude of the averaged PF-EPSC of sixty consecutive stimuli expressed as percentage of the baseline before conditioning. Sample PF-EPSC traces are taken before and 30 min after LTD induction. Each sample trace is the average of ten consecutive EPSCs.

mice, five cells studied blind). In the mutant mice, the conjunction protocol also induced persistent depression of PF-EPSCs (Figure 7B). The magnitudes of LTD in mutant slices, ranging from 59% to 104% (78.7% ± 4.9%, n = 9 from six mice, six cells studied blind) are comparable with those of the wild-type mice (p > 0.05, Mann–Whitney U test). Thus, LTD is apparently normal in the mutant mice.

Efficacy of PKC Inhibitor to Block LTD Is Modified

The presence of LTD in the mutant mice was unexpected, since previous studies indicated that LTD is abolished by PKC inhibitors (Linden and Connor, 1991; Hemart et al., 1995), and because PKCγ is the major PKC isoform in PCs (Huang et al., 1988; Kose et al., 1988; Saito et al., 1988; Tanaka and Saito, 1992). To examine whether the LTD in the wild-type and mutant mice is PKC-dependent, we applied a specific peptide inhibitor of PKC, PKC(19–36) (10 mM), intracellularly through the recording patch pipette. In the presence of the inhibitor, the conjunction protocol was ineffective in inducing depression of PF-EPSCs in the wild-type mice (Figure 7C). In fact, PF-EPSCs were slightly potentiated after conditioning. Amplitudes of PF-EPSCs measured during 25–35 min after conditioning ranged from 77% to 147% (107.8% ± 6.0%, n = 12 from seven mice, three cells studied blind), which was significantly different from the values obtained in the wild-type mice with control intracellular solution (compare Figure 7A with Figure 7C, p < 0.001, Mann–Whitney U test).

In contrast with wild-type slices, the application of PKC(19–36) did not completely abolish LTD of PF-EPSCs in mutant slices (Figure 7D). The amplitudes of PF-EPSCs

during 25–35 min after the conjunctive stimulation ranged from 82% to 107% (94.2% ± 2.7%, n = 11 from six mice, five cells studied blind), which was significantly smaller than that of the mutant mice with control intracellular solution (compare Figure 7B with Figure 7D; p < 0.05, Mann–Whitney U test). Furthermore, in the presence of PKC(19–36), the amplitudes of PF-EPSCs after the conjunctive stimulation differed significantly between the wild-type and mutant mice (compare Figure 7C with Figure 7D; p < 0.05, Mann–Whitney U test). These results suggest that LTD in the wild-type mice requires activation of PKC, while LTD in the mutant mice is mediated additionally by protein kinases that are more resistant to PKC(19–36).

The induction of LTD requires transient elevation of intracellular Ca^{2+} concentration mediated by the activation of voltage-gated Ca^{2+} channels (Sakurai, 1990; Konnerth et al., 1992). Hence, we tested whether Ca^{2+} currents in PCs were normal in the mutant mice. Whole-cell membrane currents in response to voltage steps were recorded from wild-type and mutant PCs in cerebellar slices from young (5- to 6-day-old) mice. Young mice were used because PCs at this age are almost devoid of dendrites; therefore, a good voltage control of the cell membrane can be achieved. We found that voltage-gated Ca^{2+} currents were similar in their kinetics and amplitudes in wild-type and mutant PCs (Figures 8A and 8B), indicating that these currents are normal in the mutant PCs. Moreover, intracellularly applied PKC(19–36) had no effect on the Ca^{2+} currents in either group of PCs (Figures 8C and 8D). Thus, blockade of LTD by PKC(19–36) in the wild-type mice (Figure 8C) was not due to suppression of Ca^{2+} influx through voltage-gated Ca^{2+} channels, but due to interfering with subsequent cellular processes.

Normal Intracellular Solution
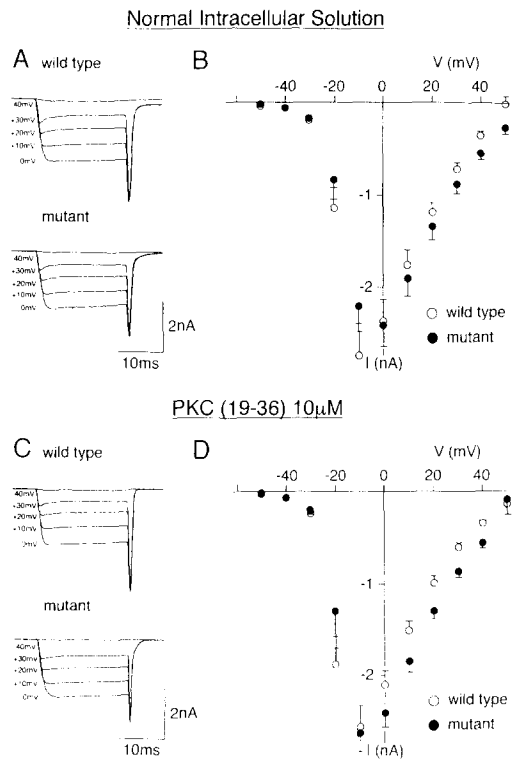

PKC (19-36) 10μM

Figure 8. Voltage-Gated Ca²⁺ Currents Are Normal in the Mutant PCs, and They Are Not Affected by the Inhibitor PKC(19-36)

(A) Whole-cell membrane currents elicited with 20 ms voltage steps from a holding potential of −60 mV to various test potentials of −40 mV, 0 mV, +10 mV, +20 mV, and +30 mV. Slices were obtained from a wild-type (P5) and a mutant (P6) mouse.

(B) Current–voltage relations for average peak Ca²⁺ currents (mean ± SEM) in wild-type (open circles, n = 10, four mice) and mutant (closed circles, n = 14, three mice) PCs.

(C and D) Voltage-gated Ca²⁺ currents were not affected by PKC(19-36). The pipette solution contained PKC(19-36) (10 mM). Sample records in (C) were obtained from a wild-type (P5) and a mutant (P4) mouse. Number of PCs in (D): wild-type, n = 8, three mice; mutant, n = 8, two mice.

Discussion

Our behavioral study indicated that PKCγ mutant mice are impaired in motor coordination, but are capable of discrete motor learning. Our electrophysiological study demonstrated that in the cerebella of these mutant mice, voltage-gated Ca²⁺ channels function normally and that LTD is induced by the standard procedure. The accompanying paper demonstrates that the innervation of multiple CFs

onto PCs persists into the adulthood in PKCγ mutant mice (Kano et al., 1995). Thus, our data suggest that an animal has a problem in conducing compound movement smoothly unless monoinnervation by CFs onto PCs is established, even if it is capable of learning component movements.

Role of PKCγ in LTD Induction

Previous results indicated that LTD induction requires activation of PKC: LTD is blocked by inhibitors of PKC (Linden and Connor, 1991; Hemart et al., 1995), and activation of PKC by phorbol esters induced depression of synaptic transmission similar to LTD (Crepel and Krupa, 1988; Crepel and Jaillard, 1991; Linden and Connor, 1991). However, the present results show that the LTD induction is intact in PKCγ mutant mice. This was unexpected, since PKCγ is the major isoform in PCs (Huang et al., 1988; Kose et al., 1988; Saito et al., 1988; Tanaka and Saito, 1992). Our study with a PKC inhibitor peptide, PKC(19-36), revealed that the intracellular mechanisms for LTD are modified in PKCγ mutant mice. PKC(19-36) totally abolished LTD in the wild-type mice, as reported previously (Linden and Connor, 1991; Hemart et al., 1995). In contrast, the same inhibitor decreased the magnitude of, but did not abolish, LTD in the mutant mice. This suggests that LTD in PKCγ mutant mice is mediated by protein kinases that are less sensitive to PKC(19-36). The δ isoform of PKC (PKCδ) has been reported to be present in the cell bodies, dendrites, and axons of PCs in the ventral folia of the cerebellum (Chen and Hillman, 1993; Merchenthaler et al., 1993). It is, therefore, possible that the lack of PKCγ is compensated by some other kinases in the mutant mice, although this isoform is used for LTD induction in the wild-type mice.

Impaired Motor Coordination Correlates with Persistent Multiple CF Innervation

Our behavioral study indicates that PKCγ mutant mice can perform classical eyeblink conditioning, but are impaired in motor coordination. This suggests that PKCγ mutant mice can acquire the memory for elements of movement, but cannot properly combine the elements spatially and temporally to achieve complex coordinated acts. Thus, PKCγ mutant mice seem to provide a unique system in which the capabilities for discrete motor learning and motor coordination can be dissociated.

To understand the underlying mechanisms, we compared the results from PKCγ mutant mice with those from

Table 1. Summary of Motor Symptoms and Cerebellar Synaptic Abnormalities

Mutant Mice	Motor Coordination	Eyeblink Conditioning	CF Innervation	LTD	Reference
PKCγ	Impaired	Facilitated	Multiple	Normal	This paper Kano et al., 1995
mGluR1	Impaired	Impaired	Multiple	Impaired	Aiba et al., 1994 Conquet et al., 1994 Unpublished data
GluRδ2	Impaired	Not tested	Multiple	Impaired	Kashiwabuchi et al., 1995
GFAP	Normal	Impaired	Normal	Impaired	Shibuki et al., submitted

three other gene knockout mice that have recently been studied and found to exhibit an impairment in either or both forms of motor behaviors (Table 1). These are mice deficient in mGluR1 (Aiba et al., 1994), the glutamate receptor δ subunit (GluRδ2) (Kashiwabuchi et al., 1995), and the glial fibrillary acidic protein (GFAP), an intermediate filament protein highly expressed in cerebellar Bergmann glia (K. Shibuki et al., submitted).

In the PKCγ mutant mice, motor coordination is impaired, and multiple CF innervation persists abnormally into adulthood. On the other hand, LTD is inducible by the standard protocol, and the animals are capable of conditioned eyeblink responses. Interestingly, mutant mice lacking GFAP exhibit an opposite phenotype. They show no sign of impairment either in motor coordination or in the elimination of surplus CFs. In contrast, LTD is deficient and eyeblink conditioning is impaired in these mutant mice (K. Shibuki et al., submitted).

Mice deficient in mGluR1 exhibit impairments in both motor coordination and eyeblink conditioning, as well as a deficiency in cerebellar LTD (Aiba et al., 1994; Conquet et al., 1994). In addition, our recent study revealed that adult mGluR1 mutant mice, like PKCγ mutant mice, retain multiple CF innervation (25% of 51 recorded PCs show multiple CF innervation; our unpublished data). Recently, some characteristics of another strain of mice deficient for GluRδ2 have been reported. These mice also exhibit motor discoordination, lack developmental elimination of multiple CF innervation (46% of 85 recorded PCs show multiple CF innervation in 4- to 6-week-old mice), and have impaired LTD (Kashiwabuchi et al., 1995). However, their capability in learning a discrete motor response (such as eyeblink conditioning) has not yet been studied.

Thus, physiological deficits and behavioral consequences observed in mutant mice with deficiency in GFAP, mGluR1, or GluRδ2 are all consistent with the notion that monoinnervation of PCs by CFs is required for motor coordination. However, it is possible that motor discoordination exhibited in these mutant mice is caused by an impairment in discrete motor learning and its putative underlying mechanism, e.g., LTD. To argue forcefully for the critical role of mono-CF innervation in motor coordination, it is important to show that the same mutant strain that exhibits motor discoordination and persistent multiple CF innervation is capable of discrete motor learning. Among the four genetically engineered mice listed in Table 1, the PKCγ strain is the only one that shows the appropriate phenotype.

LTD Is Probably a Cellular Substrate for Discrete Motor Learning

In the past, several groups studied the relevance of LTD in discrete motor learning. For instance, Ito and others found that during the adaptation of the VOR, simple spike responsiveness of floccular PCs to head rotation is progressively reduced (e.g., Dufosse et al., 1978; Watanabe, 1984; Nagao, 1991). They proposed that this reduction is a consequence of LTD at the PF–PC synapses, and hence, LTD is the key underlying cellular mechanism for this discrete motor learning (Ito, 1989, 1994). The notion that plas-

ticity at PF–PC synapses plays a role in discrete motor learning has also been incorporated into the so-called recurrent network model of the VOR by Lisberger and Sejnowski (1992), although in this model contribution of synaptic plasticity in the brainstem was also considered. Thompson and his colleagues found a class of PCs displaying a decrease in the rate of simple spike discharge in the animal undergoing eyeblink conditioning (see Thompson and Krupa, 1994). They suggested that this decrease in discharge results from LTD induced at the PF–PC synapses and that this LTD is a cellular substrate for the conditioning (Thompson et al., 1993; Chen and Thompson, 1995). The correlation observed among three gene knockout mice (mGluR1, GFAP, and PKCγ; see Table 1) between an absence or presence of LTD and an impairment or intactness of eyeblink conditioning supports the notion that LTD is a cellular substrate for discrete motor learning.

Why is eyeblink conditioning facilitated in PKCγ mutant mice that exhibit apparently normal LTD? One possibility is that the multiple CF innervation results in a higher than normal number of impulses for PCs. Under current–clamp recording conditions, we observed that each CF input of multiply innervated PCs was able to generate multiple spikes that resemble complex spikes (data not shown). During the acquisition of CRs, a higher number of complex spikes may be elicited in response to an unconditioned stimulus (US) in PKCγ mutant PCs than in wild-type PCs, and consequently, LTD induction may be facilitated in PKCγ mutant mice compared with that in the wild-type mice (Figure 9A). This may lead to a faster acquisition of CRs. The validity of this hypothesis could be tested by monitoring complex spikes in PCs that occur during eyeblink conditioning.

How Does Multiple CF Innervation Lead to Motor Discoordination?

The cerebellar cortex is composed of numerous distinct compartments, known as microzones (Oscarsson, 1979; Ito, 1984). PCs in each microzone send efferents to a small group of subcortical nuclear neurons, which in turn project to a certain subset of motor neurons. PCs in each microzone receive common excitatory inputs from CFs that emanate, in normal adult animals, from a distinct subgroup of olivary neurons in the brainstem. The cerebellar microzones and associated structures are thought to be the functional units that control component movements.

A compound movement requires a program that will coordinate many component movements in an orderly fashion (Ito, 1984; Thach et al., 1992). It is generally believed that the basic program for a compound movement, such as walking, resides in the cerebral cortex (Allen and Tsukahara, 1974; Fetz, 1993). It is also generally believed that such a basic cerebral program has to be modified or refined if the animal is to perform the movement smoothly or skillfully (Ito, 1984; Thach et al., 1992). It is likely that the cerebellum is involved in the refinement process, because animals with cerebellar lesions can generally perform compound movements but only imprecisely and unsurely (Dow and Moruzzi, 1958). However, neuroscientists differ
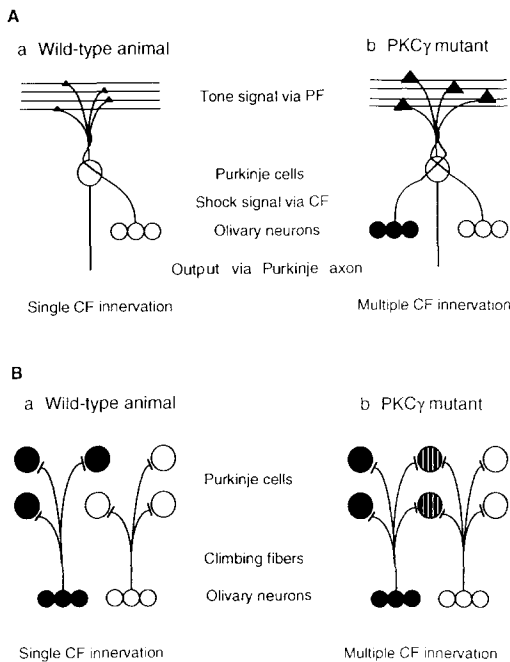
A

a Wild-type animal   b PKCγ mutant

Tone signal via PF

Purkinje cells

Shock signal via CF

Olivary neurons

Output via Purkinje axon

Single CF innervation   Multiple CF innervation

B

a Wild-type animal   b PKCγ mutant

Purkinje cells

Climbing fibers

Olivary neurons

Single CF innervation   Multiple CF innervation

Figure 9. A Model for the Role of CFs in Discrete Motor Learning and Motor Coordination

(A) Discrete motor learning. (a), CFs determine which set of PF–PC synapses should be modified and how much the synaptic strength should be. The resulting LTD at PF–PC synapses (closed triangles symbolizing LTD) presumably releases neurons in the deep cerebellar nuclei from inhibitory outputs of PCs. (b), multiple CF innervation in PKCγ mutant mice would send a larger number of CF inputs to PCs, making LTD more inducible than in the wild-type mice (larger closed triangles). Thus, eyeblink conditioning in the mutant mice is facilitated. (B) Motor coordination. (a), olivary neurons are organized into various subgroups (closed versus open circles). In the wild-type cerebellum, each PC is innervated by only one CF and thus can only be driven by one subgroup of olivary neurons at a given time in a given movement. Each functional unit of PCs (closed versus open circles) presumably corresponds to each component movement in a coordinated act. (b), in PKCγ mutant mice, certain PCs are innervated by multiple CFs. These PCs would be driven by mixed subgroups of olivary neurons (striped circles). Thus, multiple CF innervation could disrupt either the delivary of the refined motor program or the error correction mechanism through LTD induction, eventually leading to impaired motor coordination in the mutant mice.

in their views as to how the cerebellum and its associated structures accomplish the program refinement. Llinas and his colleagues argue that the refinement instruction for coordinated movements is generated in the inferior olive by dynamic synchronization of neuronal subgroups (Llinas and Yarom, 1981, 1986; Llinas and Welsh, 1993; Welsh et al., 1995). According to this idea, the role of CFs is to transmit signals for a refined program to PCs. In adult wild-type mice with monoinnervation of PCs by CFs, each PC presumably follows the direction of one subgroup of synchronous olivary neurons at a given time in a given movement. By contrast, in either adult PKCγ, mGluR1, or GluRδ2 mutant mice or wild-type mice younger than 3 weeks, certain PCs with multiple CF innervation would receive inputs from olivary neurons of two or more different subgroups. This makes it difficult for such PCs to respond in synchrony with a particular subpopulation of olivary cells

(Figure 9B). Thus, the coherence of neural activity in each cerebellar functional unit is disrupted, and the motor coordination is impaired.

Marr, Albus, and Ito argue that CFs and PCs participate more actively in the refinement of the basic motor program for a compound movement (Marr, 1969; Albus, 1971; Ito, 1984). CFs appear to transmit error signals to PCs that are detected by inferior olivary neurons as the difference between the intended and executed movements (Gellman et al., 1985; Thach et al., 1992). The error signal transmitted by a CF to a PC will induce LTD in the PF–PC synapses (Ito, 1989). This results in a suppression of the neural activity in the neural circuit responsible for the erroneous movement. In an adult wild-type animal, the error correction can be accomplished with an exquisite specificity by use of the individual cerebellar functional units that control distinct component movements. For this, the single innervation is an essential aspect of the error correction mechanism. In animals with multiple CF innervation, the organization of this cerebellar functional unit is disrupted. PCs of a microzone receive not only the proper error signal but also other signals via the surplus CFs (Figure 9B). This means that the strength of PF–PC synapses in certain microzones would be inappropriately modified by wrong error signals. As a consequence, the cerebellum cannot properly control compound movements.

If LTD is a part of the cellular mechanism that controls a component movement, why do GFAP mutant mice that lack LTD show apparently normal motor coordination (Table 1; K. Shibuki et al., submitted)? One possibility is that the employed behavioral tests for motor coordination are not sensitive enough to detect subtle impairments. Another possibility is that the LTD at the PF–PC synapses is not the only synaptic plasticity utilized for the refinement of the basic motor program. There may be another cellular substrate that may be sufficient for the smooth operation of the tested behavioral tasks.

Thus, our data are compatible with either one of the currently available hypotheses on the neural mechanism for motor coordination. What is new about the present study, particularly when combined with the recent studies on the three other mutant mice (i.e., mGluR1, GluRδ2, and GFAP; see Table 1), is that it provides an attractive hypothesis for the physiological role of the developmental elimination of the surplus CFs: monoinnervation of PCs by CFs is needed for motor coordination.

Experimental Procedures

Production of PKCγ Mutant Mice

Tests for Motor Coordination

The rotorod consists of a smooth plastic roller (8 cm diameter, 14 cm long) flanked by two large round plates (30 cm diameter) to prevent animals from escaping. A mouse was placed on the roller, and the

time remained on the stationary or rotating roller was measured. A maximal 60 s was allowed for each animal for all motor skill tests. The thin rod consists of a smooth plastic rod (1.5 cm diameter, 50 cm long) held horizontally on both ends. A mouse was placed in the midpoint of the rod, and the time remained on the rod was measured.

Footprints were made with waterproof red ink and watercolor paper. Ink was applied to the hind paws of individual mice. Mice were induced to walk forward in a narrow alley (10 cm wide, 15 cm high). Three wild-type and three mutant mice were used in footprinting.

Eyeblink Conditioning and Analysis

We utilized the eyeblink conditioning techniques developed by Skelton (1988) for rats (also Stanton et al., 1992) and adapted by Aiba et al. (1994) for mice, with minor modifications. Under ketamine (80 mg/kg intraperitoneal) and xylazine (20 mg/kg, intraperitoneal) anesthesia, animals were implanted with four Teflon-coated stainless steel wires (bare, 0.003 in; coated, 0.0045 in; A-M Systems, Incorporated, Everett, Washington) subcutaneously to their left upper eyeblink. Two of the wires were used to record differential EMG from the obicularis oculi, and the other two wires were used to deliver periorbital shock. A four-pin strip connector to which the wires were soldered was cemented to the skull of the animal with dental acrylic.

Training took place in a small cylindrical Plexiglas container (diameter, 3.75 in) that was placed inside a sound- and light-attenuating chamber (Ralph Gerbrands Company, Arlington, MA). The strip connector was attached to a mating plug of a commutator that has channels to relay EMG signal and to deliver shock. After 1 day of habituation, animals underwent 7 consecutive days of paired training. The daily paired training consisted of 100 trials grouped in ten blocks. Each block included one CS-alone (the first trial), one US-alone (the sixth trial), and eight CS-US paired trials. The CS and US were a 352 ms tone (1000 Hz, 80 dB, 5 ms rise/fall time) and a coterminating 100 ms shock (1.5 mA, 100 Hz biphasic square pulses), respectively, with a 252 ms interstimulus interval and pseudo-randomized intertrial interval between 20 and 40 s (mean, 30 s). Following paired trials, animals received 4 consecutive days of CS-alone extinction trials.

For CR analysis, the EMG signal was amplified in the band of 300–5000 Hz and sent to a window discriminator, where the number of pulses above the noise envelope was sampled and stored by a computer into 189 consecutive 4 ms bins. The discriminated EMG activity was analyzed trial by trial according to the following criteria. First, the trial was considered bad and excluded from analysis if, during the 252 ms pre-CS period, the average unit count per bin was higher than 2 (reflecting high EMG activity prior to the CS onset), or the standard deviation (SD) of unit counts per bin was larger than the average unit count per bin (reflecting unstable EMG activity prior to the CS onset), or the average unit count within 28 ms (7 bins) after the CS onset was bigger than the average plus the SD of the pre-CS period (reflecting short latency startle response to the CS). Second, the CR was defined as average unit count of any consecutive 7 bins during the period from 84 ms after tone onset to the onset of the shock (total of 168 ms) that was higher than average plus SD plus 1 unit per bin of pre-CS period. A constant value of 1 unit per bin was included as a CR threshold. This value is comparable to the CR threshold value (0.5 mm deflection in the eyeblink/nictitating membrane response) used in the rabbit eyeblink conditioning paradigm. On tone-alone trials, the period for scoring a CR was extended to the termination of the tone (total of 268 ms).

The UR amplitudes were determined from US-alone trials. Because the US (shock) saturates the EMG activity and because the shock offset is not instantaneous (owing to capacitance), we analyzed the unit activity during the 100 ms (25 bins) period 50 ms after the termination of the US. The UR amplitude was defined as the difference in averaged unit activity during the UR period and averaged unit activity 100 ms before the US onset.

Electrophysiology

Cerebellar slices were prepared from the wild-type and mutant mice as described previously (Edwards et al., 1989; Llano et al., 1991; Kano and Konnerth, 1992; Aiba et al., 1994). Either sagittal or transverse cerebellar slices of 200–300 μm thickness were cut with a vibroslicer and kept at 32°C for at least 1 hr in a chamber containing standard saline (see below) that was bubbled with 95% O_2 and 5% CO_2. One slice was then transferred to a recording chamber, where it was contin-

uously perfused with the oxygenated standard saline. Recognition of layers within the cerebellar cortex and identification of PCs were easily achieved on slices when they were viewed with a 40 × water immersion objective attached to a Zeiss upright microscope (Axioscope) (Edwards et al., 1989; Llano et al., 1991). All experiments were performed with whole-cell configuration of the patch-clamp technique with borosilicate pipettes (resistance of 3–6 MΩ when filled with an intracellular solution; see below). Ionic currents were recorded with an EPC-9 patch-clamp amplifier (HEKA) and stored on a digital audiotape (DAT) data recorder (Sony, PC204, Japan) for later analysis. The pipette access resistance was compensated as described by Llano et al. (1991). Data acquisition and analysis were performed by use of the PULSE program on a Macintosh computer (version 7.5, HEKA). The signals were filtered at 3 kHz and digitized at 20 kHz. For stimulation of CFs and PFs, a glass pipette with 5–10 μm tip diameter filled with standard saline was used. Square pulses (duration, 0.1 ms; strength, 0–100 V for CF stimulation, 1–10 V for PF stimulation) were applied for focal stimulation.

To induce LTD, we used a conjunction protocol that is composed of 240 PF stimuli in conjunction with a depolarizing pulse (200 ms, −60–+0 mV) repeated at 1 Hz. Conjunctive stimulation was applied after stable recording of PF-EPSCs for 10 min. Passive membrane properties of PCs were monitored by applying 5 mV hyperpolarizing pulses. Series resistance was monitored intermittently every 3 min and was kept constant around 12–20 MΩ.

The composition of standard saline was as follows: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1.25 mM $NaH2PO_4$, 26 mM $NaHCO_3$, and 20 mM glucose, which was bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Bicuculline (10 μM) was always present in the saline to block spontaneous inhibitory postsynaptic currents (Konnerth et al., 1992; Kano and Konnerth, 1992). For measuring voltage-gated Ca^{2+} currents, tetrodotoxin (TTX, 0.5 mM) and tetraethyl-ammonium (TEA, 75 mM) were included to block Na^+ and K^+ currents, and the concentration of NaCl was reduced to 50 mM to keep the osmolarity constant. The standard pipette solution contained 30 mM KCl, 90 mM K D-gluconate, 4 mM $MgCl_2$, 4 mM Na-ATP, 0.4 mM Na-GTP, and 50 mM HEPES (pH 7.3, adjusted with KOH). In experiments for measuring voltage-gated Ca^{2+} currents and CF EPSCs, a Cs-based pipette solution was used: 60 mM CsCl, 30 mM Cs D-gluconate, 20 mM TEA-Cl, 20 mM BAPTA, 4 mM $MgCl_2$, 4 mM ATP, and 30 mM HEPES (pH 7.3, adjusted with CsOH). All experiments were carried out at a bath temperature of 32°C, except those for voltage-gated Ca^{2+} currents, which were performed at room temperature.

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