

Subcortically and Callosally Projecting Neurons are Distinct Neuronal Pools in the Motor Cortex of the *Reeler* Mouse

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Subcortically projecting neurons and callosally projecting ones are distinct neuronal pools in the cerebral cortex of the rodents. However, cortical efferent neurons are known to project multiple targets transiently by plural collateral axons. These plural axons are eliminated during prenatal and postnatal development. In the cerebral cortex of the *Reeler*-deficient mouse, *reeler*, which is caused by mutation of the *reelin* gene, cortical efferent neurons are ectopically distributed. However, it is still unknown whether cortical efferent neurons in the *reeler* mouse lose surplus collateral axons or maintain them during developmental periods. If surplus collaterals of malpositioned cortical neurons are not eliminated, neurons projecting subcortically may project their axons to the contralateral hemisphere. To test this plausible hypothesis, we made double injections of two fluorescent dyes, Fast Blue and Diamidino yellow dihydrochloride into two of three regions, *i.e.*, upper cervical cord, ventral lateral thalamic nucleus, and contralateral motor cortex of the normal and *reeler* mice, to label corticospinal, corticothalamic and callosal commissure neurons in the motor cortex, retrogradely. No double labeled neurons were identified in the motor cortex of the normal and *reeler* mice, although the distribution patterns of these cortical efferent neurons were completely different between normal and *reeler* mice. These findings strongly suggest that collateral elimination of cortical efferent neurons during developing periods are not affected in this mutant mouse.

The mammalian cerebral cortex comprises highly orchestrated six-layered structure, and each layer is defined by a distinct morphology with specific functional properties and connections (Rakic et al., 2007; Schubert et al., 2007; Kanold and Luhmann, 2010). The laminar structures are generated by the regulated neurogenesis, appropriate neuronal differentiation, and spatiotemporally coordinated neuronal migration (Ayala et al., 2007; Sekine et al., 2011). *Reelin-Dab1* signaling pathway has been well-known to play crucial role in layer formation during brain development (D'Arcangelo et al., 1995; Katsuyama and Terashima, 2009). *Reelin* is a large secreted extracellular matrix protein that binds to

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ApoER2 and VLDLR (D'Arcangelo et al., 1995; 1999; Weeber et al., 2002; Benhayon et al., 2003; Trotter et al., 2011), and this molecular interaction induces Src family kinase-mediated phosphorylation of a cytoplasmic scaffold protein Dab1 (Arnaud et al., 2003; Bock and Herz, 2003) which binds to intracellular domain of ApoER2 and VLDLR. The *reeler* mouse, a spontaneously occurring mutant mouse, which is caused by mutated *reelin* gene, is characterized by disrupted cerebral and cerebellar cortices. In the cerebral cortex of this mutant, layer 1 is absent and corresponding cortical layers are roughly reversed (Caviness, 1976; 1982; Caviness and Sidman, 1973). For example, corticospinal (CS), corticothalamic (CT) and callosal commissure (CC) neurons are radially dispersed (Terashima et al., 1983, 1985; Inoue et al., 1991; Hoffarth et al., 1995; Yamamoto et al., 2003, 2009). In addition to *reeler*, other Reelin signal deficient mutants including Dab1-deficient *yotari*, and VLDLR/ApoER2 double knockout mice exhibit almost identical laminar disorganization in cerebral and cerebellar cortices, and hippocampus (Yoneshima et al., 1997; Howell et al., 1997; Trommsdorf et al., 1999; Hack et al., 2007).

It has long been believed that cell proliferation and differentiation of cortical neurons are not affected by abnormal neuronal cytoarchitecture in *reeler*. However, there is poor understanding about the cell fate definition of malpositioned neurons in the cerebral cortex of *reeler*. It is well known that cortical efferent neurons project extra collateral axons to multiple targets, but these extra collaterals are eliminated during developmental periods (O'Leary, 1987; Oudega et al., 1994). If these extra collaterals are not eliminated during developmental periods, cortical efferent neurons may project to surplus targets in addition to their normal target. In the normal cortex, CS, CT and CC neurons are quite distinct neuronal pools (Catsman-Berrevoets et al., 1980). If these cortical neurons maintain extra collaterals instead of eliminating them, subcortically projecting neurons and callosally projecting neurons may be partially overlapped instead of being affiliated with distinct neuronal pools. To test this hypothesis, we injected two different fluorescent dyes into two areas among three targets, *i.e.*, spinal cord, thalamus and contralateral motor cortex to label CS, CT and CC neurons retrogradely and examined whether these neurons are affiliated with distinct neuronal pools or partially overlapped.

MATERIALS AND METHODS

1. Mice

The classical *reeler* heterozygous (*Reln*^{rl/+}) mice were originally purchased from the Jackson laboratory (Bar Harbor, Me) and raised in our animal facility. The *reeler* mutant mice (*Reln*^{rl/rl}) and littermate wild type (control: *Reln*^{+/+}) were obtained from the mating with *reeler* heterozygous male and female mice. All animals were housed in temperature-controlled (22 ± 0.5 °C) colony room with a 12 hour light/dark cycle in groups in acrylic cage with free access to food and water. All experiments were carried out with the approval of the Committee on Animal Care and Welfare, Kobe University Graduate School of Medicine.

2. Tracer Injection

For the retrograde labeling, we used two neuronal tracers, Fast Blue (FB) (Sigma-Aldrich, # F3378) and Diamidino yellow dihydrochloride (DY) (Sigma-Aldrich, #D0281). FB is incorporated into the cytoplasm, while DY is incorporated into the cell nucleus. The combination of these substances makes possible fluorescent double labeling of projection neurons (Verburgh et al., 1990; Coizet et al., 2007). All adult animals mice (8-10 week of age) were anesthetized with 3.5 % chloral hydrate (1ml/100g body weight) and clamped in a

stereotactic apparatus (Narishige Co., Tokyo). Animals were injected with 0.2 μ l of 2.5% FB in distilled water (DW) and 0.2 μ l of 2% DY in DW by pressure through a glass micropipette attached to the barrel of 1 μ l Hamilton syringe under an operating microscope as described previously (Yamamoto et al., 2003). The coordinates were defined referring to the mouse brain in stereotaxic coordinates (Paxinos and Franklin, 2001). After the injection, the glass micropipette was kept *in situ* for 5 min and slowly retracted. For retrograde labeling of CC neurons, a small burr hole was made at right parietal bone above the surface of the hindlimb area (HL) of the motor cortex with a dental drill following the incision of skin, and a single injection of FB or DY was carried out (0.5 mm caudal to the bregma, 1.0 mm lateral to the midline, 0.5 mm depth from the surface of the *pia mater*). For retrograde labeling of CS neurons, the dorsal aspect of the cervical spinal cord was exposed after removal of the cervical vertebral arches, and 0.1 μ l of FB was injected into the upper cervical cord. For retrograde labeling of CT neurons, single injection of DY into the right ventral lateral thalamic nucleus (2.0 mm caudal to the bregma, 1.5 mm lateral to the midline, 2.5 mm depth from the surface of the *pia mater*).

In the present study, injections of FB and DY were simultaneously made into (1) the upper cervical cord and thalamus to label CS and CT neurons, respectively (CS/CT experiment; normal=4, *reeler*=6), (2) the contralateral motor cortex and the thalamus to label CS and CC neurons, respectively (CS/CC experiment; normal=5, *reeler*=8), and (3) the contralateral motor cortex and the thalamus to label CC and CT neurons, respectively (CC/CT experiment; normal=4, *reeler*=7).

3. Tissue preparation

The animals were deeply anesthetized with 3.5% chloral hydrate (1 ml/100 g body weight) and sacrificed by transcardial perfusion of 0.1 M phosphate buffer (PB) containing 0.9% NaCl (phosphate-buffered saline, PBS) for 5 min at room temperature, followed by 4 % paraformaldehyde in PB for 15 min at 4 °C. The brains were removed from the skull, and then post-fixed in 4 % paraformaldehyde for 2 h at 4 °C. After the post-fix, the tissues were immersed in 20% sucrose in PB for 1 day at 4 °C. The serial sections were cut coronally at 40- μ m-thickness on a freezing microtome (Yamato Koki, Osaka). The sections were mounted on gelatin-coated slides and coverslipped with FluoroGuard (Bio-Rad). The sections were observed under an Olympus AX80 microscope equipped with an UV filter system for FB- and DY-labeled neurons. The images were captured from the microscope equipped with a DP70 Digital CCD camera connected with a Windows computer using DP70 controller software (Olympus). To create photomicrographs, captured images were transferred to Macromedia Flash MX (Adobe).

RESULTS

Before describing the main results, we need to explain briefly the laminar structures of the *reeler* neocortex. In the *reeler*, no clear layer corresponding to layer I of the normal cortex is identified. The *reeler* neocortex consists of 4 layers, *i.e.*, the polymorphic cell (PM) layer, large pyramidal cell (LP) layer, granule cell (G) layer and medium and small pyramidal cell (MP+SP) layer from superficial to deeper depths (Rouvroit and Goffinet, 1998; Dekimoto et al., 2010). The PM layer occupies the most superficial layer, and corresponds to layer VI of the normal cortex. The LP layer is the second cortical layer just beneath the PM layer, and corresponds to layer V of the normal cortex. The G layer is the third cortical layer below the LP layer and corresponds to layer IV of the normal cortex. The MP+SP layer is the deepest layer and corresponds to layer II/III of the normal cortex.

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However, the borders among these cortical layers are blurred and neuronal components of these layers are distributed beyond one layer to the adjoining layers (Terashima et al., 1983, 1985; Yamamoto et al., 2003, 2009).

1. Simultaneous injection of FB into the spinal cord and DY into the thalamus (CS/CT experiment)

Figure 1 shows the simultaneous injections of FB and DY into the upper cervical cord and the ventral lateral thalamic nucleus, respectively (Fig. 1E-H). Both in the normal and *reeler* mice, FB injection was confined to the upper cervical cord, and DY injection was mainly located within the lateral ventral thalamic nucleus both in the normal and *reeler* mice. No spread of DY into the internal capsule was observed. FB-labeled neurons were recognized in the cerebral cortex, hypothalamus, red nucleus, Cajal interstitial nucleus, deep

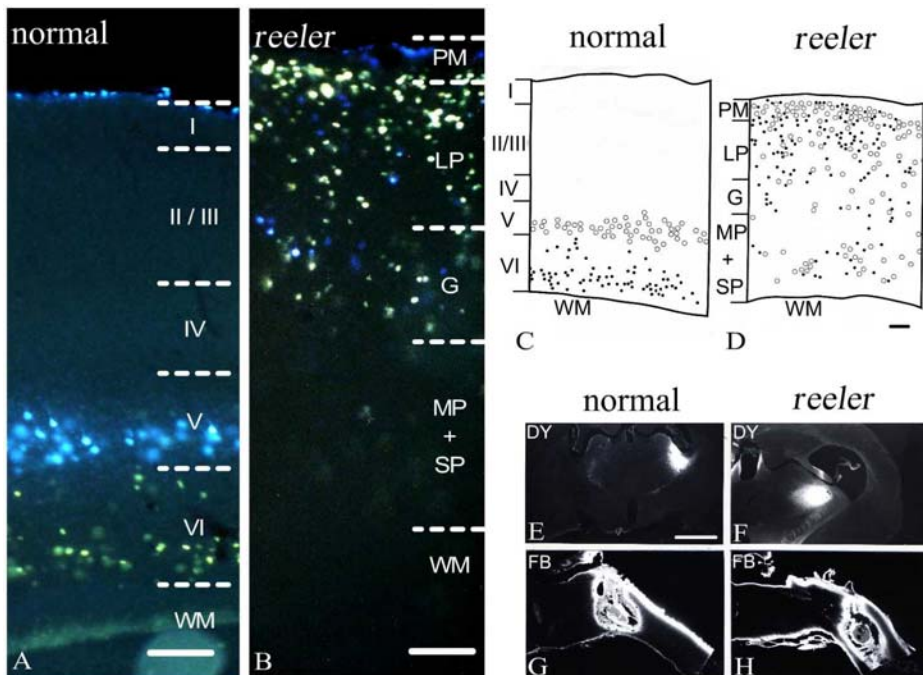


Figure 1. Distribution of Fast Blue (FB)-labeled corticospinal (CS) neurons and Diamidino yellow dihydrochloride (DY)-labeled corticothalamic (CT) neurons after the simultaneous injection of FB into the upper cervical cord and DY into the thalamus. **A, B:** In the normal motor cortex (**A**), FB-labeled CS neurons (blue) are exclusively distributed in layer V, and DY-labeled CT neurons (yellow) distributed in layer VI. The *reeler* motor cortex consists of the polymorphic cell layer (PM), large pyramidal cell layer (LP), granule cell layer (G) and medium and small pyramidal cell layer (MP+SP) from the superficial to deep, although the borders between cortical layers are blurred. In the *reeler* motor cortex (**B**), FB-labeled CS neurons are radially scattered. DY-labeled CT neurons are also radially scattered with a tendency that more neurons occupy the upper half of the cortex. **C, D:** A pair of schematic illustrations to plot FB-labeled CS neurons (open circle) and DY-labeled CT neurons (filled circle) in the motor cortex of the normal (**C**) and *reeler* (**D**) mice. No double labeled neurons are identified. **E-H:** DY was injected into the ventral lateral thalamic nucleus of the normal (**E**) and *reeler* (**F**) mice, whereas FB was injected into the upper cervical cord of the normal (**G**) and *reeler* (**H**) mice. Scale bars, 100 μm (**A-D**), 1 mm (**E-H**). Coronal (**A, B, E, F**) and sagittal (**G, H**) sections.

layers of the superior colliculus, vestibular nuclei and pontine and medullary reticular formations both in the normal and *reeler* mice (data not shown). All of these areas are known to give spinal descending pathways (Terashima et al., 1984). In the cerebral cortex of the normal mouse, FB-labeled CS neurons were exclusively distributed in layer V of the motor and somatosensory cortices. Figure 1A shows that labeled CS neurons have large pyramidal somata with an upright apical dendrite. DY-labeled CT neurons were distributed in the layer VI of the motor cortex of the normal mouse. Since DY labels nuclei but does not cytoplasm of labeled neurons (Coizet et al., 2007), no data about the morphology of DY-labeled neurons was available. FB-labeled neurons and DY-labeled neurons were plotted on the line drawing of the motor cortex (Fig. 1C, D), indicating that no double-labeled cells were identified both in the normal and *reeler* mice.

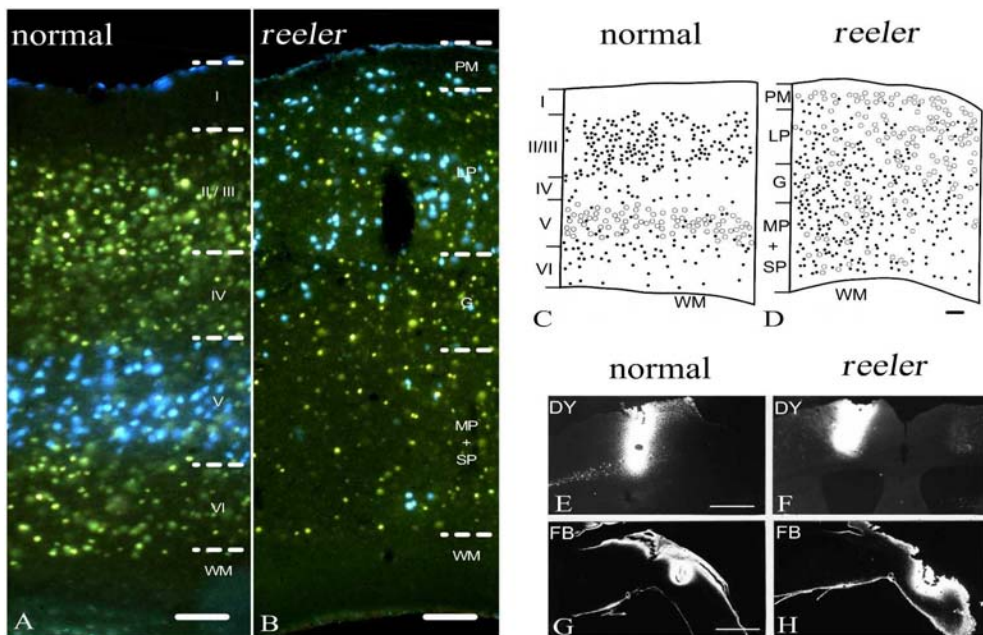


Figure 2. Distribution of FB-labeled CS neurons and DY-labeled CC neurons in the motor cortex of the normal and *reeler* mice after the simultaneous injection of FB into the upper cervical cord and DY into the corresponding motor cortex on the contralateral side. **A, B:** In the normal motor cortex (**A**), FB-labeled CS neurons (blue) are exclusively distributed in layer V, whereas DY-labeled CC neurons (yellow) are widely distributed throughout layers II-VI except for layer I with the heaviest localization in layers II/III. In the *reeler* motor cortex (**B**), FB-labeled CS neurons are radially scattered from the pial surface to the white matter. DY-labeled CC neurons are also radially scattered with a tendency that more neurons occupy the lower half of the cortex. **C, D:** A pair of schematic illustrations to plot FB-labeled CS neurons (open circle) and DY-labeled CC neurons (filled circle) in the motor cortex of the normal (**C**) and *reeler* (**D**) mice. No double labeled neurons are identified. **E-H:** DY was injected into the contralateral motor cortex (hindlimb area) of the normal (**E**) and *reeler* (**F**) mice, whereas FB was injected into the upper cervical cord of the normal (**G**) and *reeler* (**H**) mice. Scale bars, 100 μ m (**A-D**), 500 μ m (**E, F**), 1 mm (**G, H**). Coronal (**A, B, E, F**) and sagittal (**G, H**) sections.

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2. Simultaneous injection of FB into the spinal cord and DY into the contralateral motor cortex (CS/CC experiment)

Figure 2 shows the simultaneous injections of FB and DY into the upper cervical cord and contralateral motor cortex, respectively (Fig. 2E-H). Both in the normal and *reeler*, FB injection was confined to the upper cervical cord, and DY injection was located in the motor cortex (HL) on the contralateral side. DY-injection site was spread into the white matter, but not into the subcortical nuclei. FB-labeled CS neurons and DY-labeled CC neurons were observed in the motor cortex of normal and *reeler* mice (Fig. 2A, B). In the normal mouse, FB-labeled CS neurons were again exclusively distributed in layer V, whereas DY-labeled CC neurons were widely distributed from layer II to layer VI (Fig. 2A). The majority of DY-labeled CC neurons was confined to layer II/III and layer VI. In the *reeler*, FB-labeled CS neurons were dispersed from the pial surface to the white matter (Fig. 2B). More than two thirds of labeled CS neurons were distributed in the outer two thirds of the *reeler* motor cortex which corresponds to the PM layer and LP layer. DY-labeled CC neurons were widely

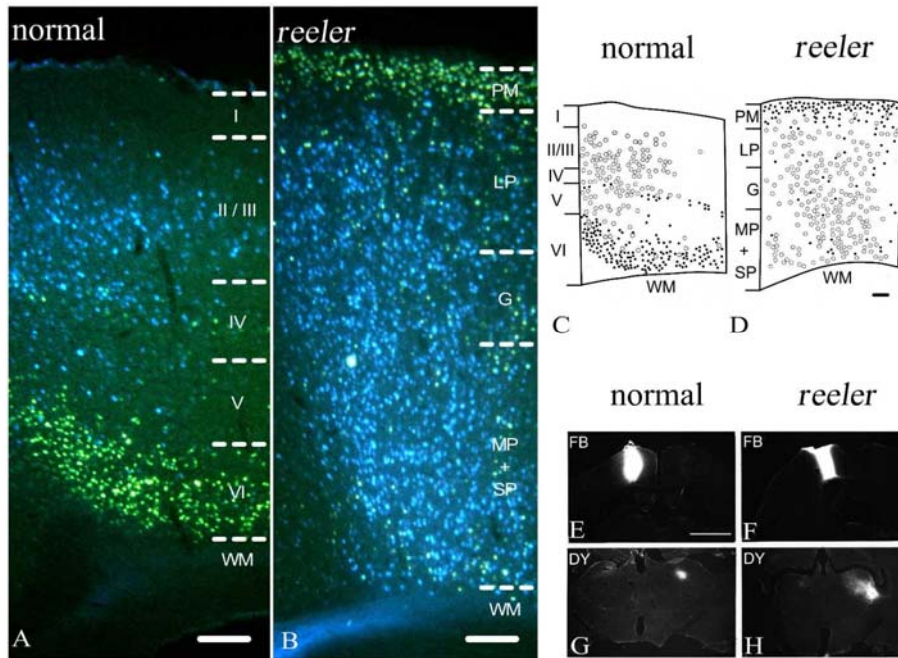


Figure 3. Distribution of FB-labeled CC neurons and DY-labeled CT neurons after the simultaneous injections of FB into the contralateral motor cortex and DY into the thalamus. **A, B:** In the normal motor cortex (**A**), FB-labeled CC neurons (blue) are radially distributed throughout the cortex except for layer I, and DY-labeled CT neurons (yellow) are mainly distributed in layer VI and occasionally found in layer V. In the *reeler* motor cortex (**B**), FB-labeled CC neurons are radially scattered, but more FB-labeled CC neurons occupy the lower half of the cortex corresponding to the MP+SP. DY-labeled CT neurons are heavily concentrated just beneath the pial surface corresponding to the PM, although a few of them are scattered in deeper layers. **C, D:** A pair of schematic illustrations to plot FB-labeled CS neurons (open circle) and DY-labeled CT neurons (filled circle) in the motor cortex of the normal (**C**) and *reeler* (**D**) mice, indicating that no double labeled neurons are identified. **E-H:** FB was injected into the contralateral motor cortex (hindlimb area) of the normal (**E**) and *reeler* (**F**) mice, whereas DY was injected into the ventral lateral thalamic nucleus of the normal (**G**) and *reeler* (**H**) mice. Scale bars, 100 μm (**A-D**), 1 mm (**E-H**). Coronal sections.

distributed throughout all of the layers of the *reeler* motor cortex. Majority of labeled CC neurons were distributed in the lower half of the cortex corresponding to the MP+SP layer. No double labeled cells were identified both in the normal and *reeler* mice (Fig. 2C, D).

3. Simultaneous injection of FB into the contralateral motor cortex and DY into the thalamus (CC/CT experiment)

The simultaneous injections of FB and DY were made into the contralateral motor cortex (HL) and the ipsilateral thalamus (Fig. 3E-H). Both in the normal and *reeler*, injection areas of FB were mainly confined to the motor cortex, but spread into the adjoining white matter. No spread into the underlying subcortical nuclei was recognized. Injection site of DY was mainly confined to the ventral lateral thalamic nucleus both in the normal and *reeler*. No spread of DY into the internal capsule occurred. In the normal motor cortex (HL), FB-labeled CC neurons were radially distributed from layer II to layer VI, and the two-thirds of labeled CC neurons were distributed in layer II/III (Fig. 3A). DY-labeled CT neurons were mainly distributed in layer VI, but a few of labeled neurons were also recognized in layer V. In the *reeler*, FB-labeled CC neurons were widely distributed from the pial surface to the white matter, but more than two-thirds of labeled cells were confined to the lower half of the motor cortex which corresponds to the MP+SP layer (Fig. 3B). DY-labeled CT neurons were radially scattered, but the majority of labeled cells were condensed just beneath the pial surface which corresponds to the PM layer. Double labeled neurons were not demonstrated both in the normal and *reeler* mice (Fig. 3C, D).

DISCUSSION

The present study has demonstrated that retrogradely labeled CT neurons after injection of DY into the thalamus of the normal mouse were mainly distributed in layer VI and only a few of labeled CT neurons occupied layer V, whereas the similar injection in the *reeler* produced radially scattering of labeled CT cells with the heaviest distribution in the PM layer just beneath the pial surface. The injection of FB into the upper spinal cord of the normal mouse resulted in retrograde labeling of CS neurons in layer V in the normal cortex, but the similar injection in the *reeler* spinal cord resulted in retrograde labeling of CS neurons that were radially scattered from the pial surface to the white matter. The injection of DY or FB into the contralateral motor cortex of the normal mouse resulted in wide distribution of retrograde CC neurons scattering from layer II to layer IV. Labeled CC neurons showed a bilaminar distribution: the highest peak of labeled CC neurons corresponding to layer II/III and the second peak corresponding to layer VI. In the *reeler*, the similar injection of dyes into the contralateral motor cortex produced wide scattering of retrograde labeled CC neurons from the pial surface to the white matter, but majority of retrogradely labeled CC neurons occupy the lower half of the motor cortex corresponding to the MP+SP layer. These findings are in accordance with the previous studies of the normal (Feig, 2004) and *reeler* (Terashima et al., 1983, 1985; Hoffarth et al., 1995; Yamamoto et al., 2003; 2009). Taken together with previous and present studies, FB and DY have high labeling efficacy for retrograde labeling of cortical efferent neurons, and therefore, combination of FB and DY used in the present study is suitable for double retrograde labeling studies of cortical efferent neurons both in the normal and *reeler* mice.

The present study has revealed that the simultaneous injections of FB into the upper cervical cord and DY into the thalamus of the normal and *reeler* mice resulted in no double labeling. In addition, double labeled cells were neither demonstrated by the simultaneous injections of FB into the upper cervical cord and DY into the motor cortex nor by the

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simultaneous injections of FB into the contralateral motor cortex and DY into the thalamus both in the normal and *reeler* mice. These findings strongly suggest that CT, CS and CC neurons are quite distinct neuronal pools both in the normal and *reeler* cortex, indicating that malposition of cortical neurons does not affect the process of elimination of surplus collateral axons during developmental periods. It has long been believed that individual cortical neurons send collaterals to both subcortical targets and the contralateral cortex (*e.g.*, Ramon y Cajal, 1894). However, electrophysiological and morphological studies failed to demonstrate such a population, suggesting that CC neurons and subcortically projecting neurons (*i.e.*, CS and CT neurons) are connectionally distinct classes of neurons (Catsman-Berrevoets et al., 1980; Swadlow and Weyand, 1981). The *postmortem* Dil labeling study demonstrated that CC neurons rarely project their axonal collaterals into the internal capsule during developing periods, suggesting that the distinction between CC neurons and subcortically projecting neurons are inherent to them and is likely to be determined at an early stage of cortical development, prior to neuronal migration from the ventricular zone (Koester and O'Leary, 1993). Recently, Gao et al. (2010) reported that Reelin-Dab1 signaling pathway plays a key role not only in cell positioning, but also in maintenance of neuronal progenitor cells and cell differentiation. Thus, it should be expected that the fate of axonal collaterals may be changed in *reeler*. The present double labeling study, however, failed to demonstrate that surplus axonal collaterals of *reeler* cortical efferent neurons are not eliminated, but maintained.

In conclusion, in the *reeler*, cortical efferent neurons projecting their axons to the contralateral hemisphere and those projecting axons subcortically to the thalamus or the spinal cord are distinct neuronal pools, as is the case of the normal control, and therefore, malpositioned cortical efferent neurons including CS, CT and CC neurons in the *reeler* motor cortex do not affect the mechanism which governs stabilization or elimination of axonal collaterals.

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