Anterograde labeling of the corticospinal tract in *jimpy* mutant mice by DiI injection into the motor cortex

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Summary. Carbocyanine fluorescent dye, DiI, is an excellent anterograde/retrograde neural tracer, but its efficacy for the anterograde labeling of neural circuits in the adult brain tends to decrease with ages. The present study shows that an injection of DiI into the motor cortex of the young adult *jimpy* mutant mice (*Plp1^{jp/+}*) resulted in successful anterograde labeling of corticospinal tract fibers. Furthermore, an injection of Fast Blue into the lumbar spinal cord of the mutant mice resulted in retrograde labeling of layer 5 corticospinal tract neurons within the motor cortex. Since no abnormality except for myelin deficiency is known in the long descending and ascending tracts of *jimpy* mutant mouse, this mutant is suitable for neural tracing studies of long axonal trajectories with the use of carbocyanine dye, DiI, although these males die between 20 and 40 days of age

Introduction

The fluorescent carbocyanine dye DiI can be used for retrograde and anterograde labeling of neuronal pathways and has proven to be a useful tool in fields of developmental neurobiology (O'Leary and Terashima 1988; Honig and Hume, 1989; Muraoka *et al.*, 2007). One of the best features of this dye is that it can label axons by lateral diffusion in the axonal membrane in fixed tissue (*postmortem* labeling) (Brehmer *et al.*, 1999), which enables the study of human neural networks (*e.g.*, Tardif and Clarke, 2001). In addition, fluorescent staining of DiI can be photoconverted into stable diaminobenzidine (DAB) products (Singleton and Casagrande, 1996). Since the DAB reaction product formed during photoconversion is electron dense, an important advantage of this application is its allowance of correlations between light and electron microscopy analyses (Nikonenko *et al.*, 2005). In spite of these advantages, the efficacy of axonal labeling by all lipophilic carbocyanine dyes gradually decreases in advance of myelination during the postnatal days, and sufficient axonal labeling in the mature CNS cannot be anticipated. The reason why axonal labeling becomes less complete as myelination increases with age remains to be determined.

The mouse mutant *jimpy* carries an X chromosome-linked recessive gene defect that affects the formation of myelin in the central nervous system (Gotow *et al.*, 1999). A single nucleotide difference at a splice site in the gene (*Plp1*: proteolipid protein 1) for myelin proteolipid protein in *jimpy* mice results in the removal of an exon that induces myelin deficiency in the *jimpy* CNS (Macklin

Received December 20, 2007

* This study was supported by grants-in-aid from the Ministry of Science, Education and Culture and Technology to T. T. (No. 17300111, 18650093, 18021025).

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et al., 1987; Moriguchi *et al.*, 1987; Nave *et al.*, 1986). From our past experience, it became evident that DiI anterograde labeling succeeds only in brains of young animals — perhaps because myelin interferes in older ones (Terashima *et al.*, 1995). Accordingly, suitable axonal labeling of unmyelinated axons of long trajectories by DiI application might be anticipated in the CNS of the *jimpy* mouse. Therefore, in the present study, we injected DiI solution into the motor cortex of adult *jimpy* mutant mice to label corticospinal tract (CST) axons anterogradely.

Materials and Methods

Animals

Jimpy mutant mice (*Plp1^{jp/+}*) on the B6CBA/J-jp background were originally purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and kindly presented by Dr. Ikenaka of the National Institute for Physiological Sciences (Okazaki). All animals were housed in a temperature-controlled ($22 \pm 0.5^\circ\text{C}$) colony room at a 12h/12h L/D cycle in groups in acrylic cages with woodchip bedding and unlimited access to normal laboratory chow and water. All experiments were carried out with the approval of the Committee on Animal Care and Welfare, Kobe University Graduate School of Medicine.

Retrograde labeling of CST neurons

Young adult mice of 3 weeks of age (3 normal, 2 *jimpy*) were used in the present study. The animals were anesthetized with 3.5% chloral hydrate by an intraperitoneal injection (0.9 ml per 100 g body weight) and clamped in a stereotactic apparatus (Narishige, Tokyo). For retrograde labeling of CST neurons, the dorsal aspect of the upper lumbar cord of the animals was exposed after the removal of the lumbar vertebral arches, and 0.5 μl of 2.5% Fast Blue (FB) dissolved in distilled water was injected into both sides of the upper lumbar spinal segments through a glass micropipette attached to the barrel of a 1 μl Hamilton syringe under an operating microscope. Forty-eight hours after injection, the animals were deeply anesthetized with Nembutal (pentobarbital sodium, 0.2 ml per 100 g body weight) and then transcardially pre-perfused with phosphate-buffered saline (PBS) for 5 min at room temperature, perfused by 4% paraformaldehyde in a 0.1M phosphate buffer (PB; pH 7.4) for 10 min at 4°C , followed by perfusion of the same fixative containing 10% sucrose for 5 min (ice cold). The

brain and spinal cord were immediately removed from the skull and vertebral canal, respectively, and immersed overnight at 4°C (post-fixation) in the same fixative containing 30% sucrose. The brains were then sectioned coronally or sagittally at a 40 μm thickness on a freezing microtome and collected in 0.1M PB. The sections were mounted on gelatin-coated slides. The sections were examined under an Olympus AX80 microscope equipped with a UV filter.

Anterograde labeling of CST neurons

For anterograde labeling of CST axons, young adult animals of 3 weeks of age (2 normal, 3 *jimpy*) were anesthetized with 3.5% chloral hydrate by an intraperitoneal injection and clamped in a stereotactic apparatus as described above. Following an incision in the skin, a small bur hole was made in the left parietal bone using a dental drill. A single injection of 0.1 μl of 10% DiI solution dissolved in N,N-dimethylformamide was made in the left motor cortex (hindlimb area) by pressure through a glass pipette attached to the Hamilton syringe. Two days later, the animals were anesthetized with 10% Nembutal and transcardially perfused with a small volume of 0.1M PB, and then 4% paraformaldehyde in 0.1M PB for 15 min at 4°C . The brains were removed from the skull and stocked in the same fixative overnight at 4°C (post-fixation). The brains were embedded in 2% agarose and horizontally sectioned at a 100 μm thickness on a microslicer ((Dosaka-EM, Kyoto). They were mounted on MAS-coated slides (Matsunami Glass Industry, Osaka) and coverslipped with FluoroGuard (Bio-Rad). Histological sections were examined under an Olympus AX80 microscope equipped with a FITC filter.

Results

Retrograde labeling of CST neurons

Although the course of CST axons of *jimpy* mouse has been confirmed to be normal by the Golgi impregnation method (Valverde, 1966) and WGA-HRP tracing (Stanfield, 1991), the intracortical position of CST neurons of this mutant had not been examined previously. The injection of FB into the lumbar spinal cord resulted in retrograde labeling of CST neurons in the motor cortex of normal and *jimpy* mutant mice (Fig. 1). Both in the normal and *jimpy* mice, labeled CST neurons were distributed mainly in the hindlimb area (HL) although a few labeled CST neurons were also seen in the adjacent forelimb area (FL) and area 1 of the frontal cortex (Fr1).

Fig. 1. Retrogradely labeled corticospinal tract (CST) neurons after an injection of Fast Blue (FB) into the lumbar spinal cord of adult normal (**A**) and *jimpy* (**B**) mice. FB-labeled neurons with upright apical dendrites are exclusively localized within layer V of the motor cortex of the normal and *jimpy* mice.

Retrogradely filled cells were exclusively positioned in layer V both in the normal and mutant mice. Retrogradely labeled cells had pyriform somata and could be classified as large pyramidal neurons with an upright apical dendrite both in the normal and mutant mice. No differences in distribution pattern, intracortical positioning, or cellular configuration was recognized between the normal and mutant mice, suggesting the origins for CST projection are normal in the *jimpy* mice.

Anterograde labeling of CST axons

Injection of DiI into the motor cortex of the adult *jimpy* mutant resulted in anterograde labeling of CST axons at a single fiber resolution (Fig. 2). DiI-labeled axons exited from the motor cortex and entered into the internal capsule and the pyramidal peduncle, successively (Fig. 2A). They penetrated through the basal pontine gray as longitudinal pontine fibers, giving off collateral branches terminating with pontine nuclei neurons. DiI-labeled axons then entered into the medullary pyramid and crossed at the pyramidal decussation to course through the dorsal funiculus contralateral to the injection side. They ran rostro-caudally in the ventralmost area of the dorsal funiculus and gave off terminal branches in the anterior horn and the intermediate substance (Fig. 2A). The course of CST axons and their terminal branches were identical to the normal counterparts, suggesting that the course of CST axons of *jimpy* mouse and their terminals continue to be normal in spite of a myelin deficiency. In conclusion, *jimpy* CST axons were completely filled with DiI, and

suitable labeling was obtained at a single-fiber resolution in a similar manner to the DiI anterograde labeling of immature CST axons of the normal pups.

Discussion

The present study has demonstrated that CST axons of the adult *jimpy* mice can be anterogradely labeled at a single fiber resolution. The lipophilic fluorescent dye DiI is an excellent anterograde tracer due to its high fluorescence intensity, resistance to photobleaching, and non-cytotoxicity (Vercelli *et al.*, 2000). Furthermore, the DiI labeling method is technically simple, both in its tracer application and tissue processing. However, the efficacy of DiI labeling of long trajectories decreases with aging, and sufficient labeling of axons at a single fiber resolution cannot be obtained when this dye is applied to the adult CNS. In order to overcome this demerit for DiI, we previously tried a long survival experiment of more than three weeks after the application of DiI into the motor cortex of the adult rat, which resulted in favorable anterograde labeling of mature CST axons (Terashima *et al.*, 1995). The present study employing a myelin-deficient mutant mouse *jimpy* proposes another method of DiI neural tracing of adult projections. Although the reason why DiI can move over a long distance to fill the mature axons in the adult *jimpy* CNS remains unknown, the present study suggests that a myelin-deficient mutant mouse, *jimpy*, may serve as an experimental animal suitable for DiI tracing of mature long fiber trajectories.

Fig. 2. Anterogradely labeled CST axons of the adult *jimpy* mouse after the injection of DiI solution into the left motor cortex (hindlimb area). **A:** Sagittal section of the brainstem ipsilateral to the injection site. DiI-labeled CST axons enter the cerebral peduncle (cp) and then penetrate the basal pontine nuclei (Pn) to project pontine collaterals. They enter the medullary pyramid (py), descend to the ventral surface of the medulla, and cross at the pyramidal decussation (pyx) to the contralateral spinal cord. **B:** Coronal section of the lower cervical cord. The DiI-labeled axons descend in the ventralmost area of the dorsal funiculus contralateral to the injection site and give off many spinal collaterals into the spinal gray. Other abbreviation: IO, inferior olive.

It is a prerequisite for DiI neural tracing with a *jimpy* mutant that long fiber trajectories of this mutant mouse be normally preserved except for unmyelination. The present study has shown that the intracortical distribution of CST neurons and the course of CST axons both in the brainstem and the spinal cord are similar to their normal counterparts. Previous studies based on the rapid Golgi method (Valverde, 1966) and WGA-HRP tracing method (Stanfield, 1991) demonstrated that the course and terminal branching of *jimpy* CST axons are not affected by myelin deficiency. Taken together, these findings indicate that the *jimpy* CST system appears to be normal both in its origins and its course. To our knowledge, neither cytoarchitectural nor hodological anomalies in the *jimpy* CNS have been reported, suggesting that DiI neural tracing with this mutant is helpful in developmental, regenerative, and degenerative studies of the rodent CNS, although these mutant mice die between 20 and 40 days of age.

Acknowledgements

The authors wish to thank Dr. K. Ikenaka (National Institute for Physiological Sciences, Okazaki, Japan) for his kind gift of *jimpy* mutant mice.

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