PKH26 is an excellent retrograde and anterograde fluorescent tracer characterized by a small injection site and strong fluorescence emission

Koji Kawaguchi, Yu Katsuyama, Satoshi Kikkawa, Tomiyoshi Setsu, and Toshio Terashima

Division of Anatomy and Developmental Neurobiology, Kobe University Graduate School of Medicine, Kobe, Japan

Summary. The fluorescent dye PKH26, which binds mainly to the cell membrane, has long stability that enables the tracing of PKH26-labeled transplanted cells in host tissue. In the present study, we examined whether this fluorescent dye works as a retrograde or anterograde tracer to label neural networks within the central nervous system of adult and postnatal day 3 (P3) mice. A small injection of the dye into the medullospinal junction resulted in the retrograde labeling of corticospinal tract (CST) neurons in layer V of the sensory-motor cortex both in the adult mice and pups. Injection of the dye into the motor cortex of the P3 pups resulted in the anterograde labeling of CST fibers at a single fiber resolution level, although a similar injection of the dye into the motor cortex of the adult mice failed to stain CST fibers anterogradely. These results suggest that, while PKH26 works as a retrograde or anterograde tracer, anterograde labeling of the adult tracts can not be expected.

Introduction

In the first half of the 20th century, two pathological phenomena—the retrograde cell reaction and Wallerian degeneration—were widely utilized in experimental neuroanatomy to reveal neuronal circuits (Grant, 1970; Heimer, 1970). However, these methods using pathological signs of degeneration were complicated and the results obtained were not stable. In 1970, the neural tracing method based on axonal transport was introduced into neuroanatomy. The axon of neurons employs bidirectional flows that are driven by anterograde and retrograde transports to convey molecules involved in neuronal function. The invention of methods using these axonal transports, which are very easy and stable compared with axonal degeneration methods, accelerated the accumulation of knowledge about neural circuits in the decade of the 1970s. Since the first report of the introduction of horseradish peroxidase (HRP) to label the nervous system by LaVail and LaVail (1972), many retrograde tracers such as WGA-conjugated HRP, Fast Blue, Fluoro-Gold, and carbocyanine dyes have been developed. Among these retrograde tracers, fluorescent dyes are useful for identifying the chemical characters of labeled cells by their combinational use with fluorescent immunohistochemistry (Terashima et al., 1995). However, these retrograde tracers suffer from many disadvantages. For example, the fluorescence of Fast Blue quickly fades out under the ultraviolet illumination (Terashima et al., 1995). In addition, the injection site tends to diffuse into the surrounding area. The fluorescence of Fluoro-Gold is resistant to histological processes such as dehydration in ethanol and clearing in
xylene, but its cytotoxicity is not appropriate for a long survival experiments (Garrett et al., 1991). Carbocyanine dyes, which were originally developed as an excellent anterograde tracer (O'Leary and Terashima, 1988), are retrogradely transported to label cells of the nerve tracts (Lee et al., 2005), but a vibratome or microslicer is essential to cut brains and spinal cords instead of a freezing microtome or cryostat.

The fluorescent dye PKH26, which binds mainly to the cell membrane (Horan and Slezak, 1989; Slezak and Horan, 1989), has strong membrane retention and gives strong fluorescent emission as compared with other fluorescent dyes such as rhodamine, which binds mainly to intracellular proteins (Butcher and Weissman, 1980). Since the fluorescence of PKH26-labeled cells persists for a long time both in vivo and in vitro, PKH26 has been repeatedly used to trace the metastasis of carcinoma cells (Yamagata and Kumagai, 2000), the migration of stem cells (Tang et al., 2009), and the proliferation of transplanted mesenchymal cells in the host tissue (Secretan et al., 2010 in press). This fluorescent dye is also available for tracing the migration of neurons (Nagata and Terashima, 1994) and proliferation of neural stem cells in vitro (Bantubungi et al., 2007). However, no evaluation of this fluorescent dye as a retrograde or anterograde tracer in vivo has been undertaken yet. Thus, in the present study, we examined whether PKH26 is retrogradely or anterogradely transported in the corticospinal tract (CST) fibers of adult mice and pups.

Materials and Methods

Animals

Mice of a C57BL/6J background were originally purchased from the Jackson Laboratory and maintained in our laboratory. All animals were housed in a temperature controlled (22°C ± 2°C) colony room with a 12-h/12-h light/dark cycle in groups in acrylic cages with woodchip bedding and free access to laboratory chow and water. All animal experiments were carried out with the approval of the Committee on Animal Care and Welfare, Kobe University School of Medicine. Adult mice at 2 months of age and pups at postnatal day 3 (P3) were used in the present study.

Retrograde labeling of CST neurons

Three adult mice of 2 months of age were anesthetized with 3.5% chloral hydrate by an intraperitoneal injection (1 ml per 100 g body weight) and clamped in a stereotactic apparatus (Narishige, Tokyo). For the retrograde labeling of CST neurons, the midline of the skin of the occipital region was cut and the dorsal aspect of upper cervical cord of the animals was exposed after removal of the neck muscles. Then 0.5 µl of PKH26 solution (Zynaxis Cell Science, USA) was injected into both sides of the upper lumbar cord of the animals via a glass micropipette attached to the barrel of a 1 µl Hamilton syringe under an operating microscope. After surviving 2 days, the animals were deeply anesthetized with Nembutal (pentobarbital sodium, 0.2 ml per 100 g body weight) and killed by a transcardial perfusion of phosphate buffered saline (PBS) for 2 min at room temperature, followed by 4% paraformaldehyde in a 0.1M phosphate buffered saline (pH 7.4; PB) for 15 min at 4°C. After the perfusion, the brains and upper cervical cords were removed from the skull and vertebral canal and immersed in PB containing 30% sucrose overnight at 4°C. The brains and spinal cords were coronally or sagittally sectioned at 40 µm thickness on a freezing microtome. Sections were collected in 0.1M PB, mounted on gelatin-coated slides, and examined under a fluorescent microscope equipped with the appropriate filter system for PKH26 (Nikon, G filter, Tokyo).

To examine whether PKH26 works as a retrograde tracer in young animals, five pups at P3 age were anesthetized on ice, and 0.1 µl of PKH26 was injected into the medullospinal junction as described above. After one day of survival, the pups were sacrificed with a transcardial perfusion of the fixative in a similar way. Instead of making frozen sections, the brains and spinal cords of the P4 pups were embedded in agarose and sectioned at 50 or 100 µm thickness on a microslicer (Dosaka EM, Kyoto).

Anterograde labeling of CST axons

To examine whether PKH26 works as an anterograde tracer, we injected a small volume of PKH26 into the motor cortex (hindlimb area) of adult mice at 2 months of age and P3 pups. Four adult animals were anesthetized with 3.5% chloral hydrate and clamped in a stereotactic apparatus. After the scalp had been retracted, a small burr hole was made directly over the motor cortex (hindlimb area) under visual guidance using a map of the mouse brain (Franklin and Paxinos, 2008). In all experiments, 0.1 µl of PKH26 solution was injected under the operating microscope. The method of fixation, cryoprotection, histochemical procedures, and observation were the same as described in the retrograde labeling. To examine whether PKH26 works as an anterograde tracer in young animals, 5 pups at P3 were anesthetized...
on ice, and 0.1 µl of PKH26 was injected into the motor cortex (hindlimb area) as described above. After one day of survival, the pups were sacrificed with transcardial perfusion of the same fixative. The brain and spinal cord were respectively removed from the skull and vertebral column, and immersed in the same fixative for more than 24 h. The brain and spinal cord of P4 pups were embedded in agarose and sectioned at 50 or 100 µm thickness on a microslicer.

Results

Retrograde labeling of CST neurons

PKH26 was injected into the medullospinal junction of the adult mice. The injection site was confined to the medullospinal junction containing the pyramidal decussation (Fig. 1A, B). No spread of fluorescent dye to the adjoining areas was recognized except for that of only a few PKH26-labeled axons nearby the injection site to a short distance. The cell bodies of CST neurons retrogradely labeled by PKH26 were distributed in layer V of the sensorimotor cortex of the adult mice (Fig. 1C, D) in a similar manner as previously reported (Yamamoto et al., 2003; Kassai et al., 2008; Bilasy et al., 2009). Cell somata and apical and basal dendrites of CST neurons were filled with PKH26. Strong fluorescence emission was observed under the fluorescent microscope. Fluorescence was stable and did not quickly fade out under the excitation illumination. In addition to CST neurons, rubrospinal tract neurons, tectospinal tract neurons, and pontine and medullary reticulospinal tract neurons were retrogradely labeled (data not shown). A similar injection of PKH26 into the medullospinal junction of P3 pups resulted in the retrograde labeling of CST neurons in layer V of the sensorimotor cortex of P4 pups in a manner similar to that of the adult mice (data
that this dye is not anterogradely transported in the mature brain. However, a similar injection of dye into the P3 pups resulted in the anterograde labeling of CST axons, revealing that PKH26-labeled axons exited from the injection site, entered into the internal capsule, and passed through the cerebral peduncle. These axons penetrated through the basal pons as longitudinal fibers of the pons and descended through the pyramis of the medulla (Fig. 2).

**Fig. 2.** Anterogradely labeled CST axons. PKH26 was injected into the motor cortex of the postnatal day 3 (P3) mouse one day prior to sacrifice. A, B: PKH26-labeled CST axons penetrate into the basal pons as longitudinal fibers of the pons (lfp). PKH26 labeled CST axons give off pontine collaterals terminating at the pontine nuclei (Pn). The arrow in B shows a branching point of pontine collaterals arising from the parent axon. C, D: Labeled CST axons ascend dorsalward in the pyramidal decussation (pdx) to enter into the ventral margin of the dorsal funiculus (df) of the cervical cord (CC) contralateral to the injection site. PKH-labeled axons give off collaterals projecting to the gracile nucleus (Gr). E, F: PKH-labeled CST axons descend in the ventralmost region of the df of the spinal cord throughout the spinal cord. Photo images B, D, F are enlarged from A, C, E, respectively. Other abbreviation: gr, gracile funiculus. Sagittal sections. Rostral is left; dorsal is up. Scale bars: 100 μm (A, C, E), 50 μm (B, D, F).

Anterograde labeling of CST axons

To examine the utilization of PKH26 as an anterograde neuronal tracer, it was injected into the motor cortex (hindlimb area) of adult mice and P3 pups. CST axons were not labeled in the operated adult brain, suggesting
PKH26-labeled CST fibers turned dorsally at the medullospinal junction to accomplish pyramidal decussation and entered into the ventral-most region of the dorsal funiculus of the contralateral cervical cord (Fig. 2C, D). They descended throughout the ventral margin of the dorsal funiculus of the spinal cord (Fig. 2E, F). During the course of CST in the brain and spinal cord, labeled CST fibers gave off collateral branches projecting to the basal pontine gray (Fig. 2B, arrow) and the gracile nucleus (Fig. 2D), as previously reported (O’Leary and Terashima, 1988, Terashima, 1995a, b). PKH26-labeled CST axons were recognized at a single fiber resolution under the fluorescent microscope.

In addition to the anterograde labeling of CST axons, the PKH26 injection into the motor cortex (hindlimb area) resulted in the retrograde labeling of association neurons (Fig. 3A–C) and callosal commissure neurons (Fig. 3D). Figures 3A and 3B show that retrogradely labeled association neurons were distributed in cortical layers II–V of the ipsilateral occipital cortex, with a predominance in layers II/III. Cell somata and apical and basal dendrites of association neurons in layer V

**Fig. 3.** Commissure and callosal neurons retrogradely labeled with an injection of PKH26 into the motor cortex of a P3 pup one day prior to sacrifice. **A, B:** These two low-power photoimages show retrogradely labeled association neurons in the occipital cortex ipsilateral to the injection site. Labeled neurons are distributed in cortical layers II/III-V with a predominance in layer II/III. Axons exiting from retrogradely labeled neurons are also labeled (asterisk). **C:** This high-power photoimage is enlarged from **A.** The arrow shows the axon of a retrogradely labeled association neuron whose apical dendrite is filled with the dye. **D:** Callosal commissure neurons located in cortical layer II/III are retrogradely labeled. Axons (arrow) are also labeled. Other abbreviation: wm, white matter. Sagittal sections. Rostral is left; dorsal is up. Scale bars: 100 μm (A, B, D), 50 μm (C).
were filled with PKH26 (Fig. 3C). Axons exiting from the base of association neurons were also labeled (Fig. 3C, arrow). PKH26-labeled callosal commissure neurons were predominantly distributed in layers II/III of the contralateral hemisphere (Fig. 3D). Axons of the callosal commissure neurons were also labeled (Fig. 3D, arrow).

Discussion

The present study demonstrated that PKH26 works as an excellent anterograde and retrograde tracer although anterograde transport of this dye can not be expected in the mature brain. The fluorescent emissions of retrogradely labeled cells are very strong and stable, and the fluorescence does not fade out under long-time illumination. In addition, anterogradely labeled axons can be recognized at a single fiber resolution, enabling us to trace collaterals of PKH-labeled parent axons. These remarkable properties of PKH26 are very similar to those of the lipophilic carbocyanine dyes (e.g., DiI, DiA, DiO and 4Di-10ASP) though we did not confirm whether PKH26 can be used for postmortem labeling to label aldehyde-fixed brains and spinal cords as in the case of carbocyanine dyes (Harrison et al., 2005). PKH26 has been used to label cells, organelles, liposomes, viruses, and lipoproteins in a wide variety of long-term tracing applications, including cell transplantation, migration, adhesion, and fusion studies (Modo et al., 2009; Chang et al., 2009; Ji et al., 2009). However, the present study is the first to demonstrate that PKH26 is a particularly important dye for retrograde and anterograde tracing in neural networks.

The remarkable stability of some fluorescent tracers for retrograde neuronal labeling including Fast Blue, fluorescent microsphere, Diaminido Yellow Dihydrochloride, and Fluoro-Gold in vivo enables the observation of labeled neurons several weeks after tracer application. Among these fluorescent tracers, Fast Blue has been repeatedly used for long survival time experiments because this retrograde tracer does not show any signs of cytotoxicity against the nervous tissue and the tracer retains in labeled cells for up to a year (Asahara et al., 1999). Pettersson et al. (2010) investigated the efficiency of several fluorescent cell tracers for the long-term labeling of olfactory ensheathing glial cells in culture following their transplantation into the rat spinal cord. They demonstrated that the number of cells labeled with PKH26 was reduced to 51–55% after 2 weeks of culture and reached 8–12% after 4–6 weeks, while Fast Blue-positive cells remained unchanged during the first 4 weeks—but only about 21% cells retained the tracer 6 weeks after labeling. Based on this study, Fast Blue is more suitable than PKH26 for a study requiring a long survival period after the initial labeling of neurons. However, the injection site of Fast Blue tends to spread into the adjoining regions making it more difficult for researchers to interpret the results (Aschoff and Holländer, 1982). The present study demonstrated that PKH26 is confined to the injection site instead of diffusing to the adjoining areas. In addition, although the fluorescence of Fast Blue quickly fades out under ultraviolet illumination (Terashima et al., 1995), the strong fluorescent emission of PKH26 is stable and resistant to excitation illumination under fluorescent microscopy. These distinguishing properties of PKH26 make this dye particularly important for neural retrograde and anterograde tracers for long survival time experiments.

The disadvantages of PKH26 are as follows: 1) cryosectioning using a cryostat or freezing microtome is not suitable for using this dye as an anterograde tracer and 2) anterograde labeling can not be expected in the nervous system of the adult animals. These disadvantages of this dye are very similar to those of carbocyanine dyes (Shibata-Iwasaki et al., 2007). PKH26 and carbocyanine dyes are chemically lipophilic and retained in the lipid layer of cell membranes (Axelrod, 1979; Fischer and Mackensen, 2003; Sengupta et al., 2007). Thus, these common chemical properties may be the reason why these molecules share similar disadvantages. First, cryosectioning may cause the degradation and fragmentation of cell membranes of anterogradely labeled axons. As a consequence, the dyes dissolve out from the labeled axons and the increased tissue background conceals any fluorescence of labeled axons under the fluorescent microscope. It should be noted that cryosectioning is possible for the retrograde labeling of cell bodies of neurons both in the mature and immature nervous tissues. Second, efficacy of anterograde labeling with an use of carbocyanine dyes is rapidly decreased after initiation of myelination. However, a long survival period after Dil injection prior to sacrifice (several weeks) enables bright anterograde labeling of mature axons including their collaterals and terminals (Terashima et al., 1995). Therefore, anterograde labeling of mature axons with the use of PKH26 may be expected after longer survival periods. Alternatively, the adult jimpy mutant mouse may be suitable for neural tracing studies with the use of PKH26, because no abnormality except for myelin deficiency has been found in the neural networks of this mutant mouse (Valverde, 1966; Stanfield, 1991; Shibata-Iwasaki, et al., 2007).
References

Asahara T, Lin M, Kumazawa Y, Takeo K, Akamine T, Nishimura Y, Kayahara T, Yamamoto T: Long-term observation on the changes of somatotopy in the facial nucleus after nerve suture in the cat: morphological studies using retrograde labeling. Brain Res Bull 49: 195-202 (1999).

Aschoff A, Holländer H: Fluorescent compounds as retrograde tracers compared with horseradish peroxidase (HRP). I. A parametric study in the central visual system of the albino rat. J Neurosci Meth 6: 179-197 (1982).

Axelrod D: Carbocyanine dye orientation in red cell membrane studied by microscopic fluorescence polarization. Biophys J 26: 557-573 (1979).

Bantubungi K, Blum D, Cuvelier L, Wislet-Gendebien S, Rogister B, Brouillet E, Schiffmann SN: Stem cell factor and mesenchymal and neural stem cell transplantation in a rat model of Huntington’s disease. Mol Cell Neurosci 37: 454-470 (2008).

Bilasy SE, Satoh T, Ueda S, Wei P, Kanemura H, Aiba A, Terashima T. Kataoka T: Dorsal telencephalon-specific RA-GEF-1 knockout mice develop heterotopic cortical mass and commissural fiber defect. Eur J Neurosci 29: 1994-2008 (2009).

Butcher EC, Weissman EL: Direct fluorescent labeling of cells with fluorescein or rhodamine isothiocyanate. I. Technical aspects. J Immunol Methods 37: 97-108 (1980).

Chang YS, Oh W, Choi SJ, Sung DK, Kim SY, Choi EY, Kang S, Jin HJ, Yang YS, Park WS: Human umbilical cord blood-derived mesenchymal stem cells attenuate hyperoxia-induced lung injury in neonatal rats. Cell Transplant 18: 869-886 (2009).

Fischer K, Mackensen A: The flow cytometric PKH-26 assay for the determination of T-cell mediated cytotoxic activity. Methods 31: 135-142 (2003).

Franklin KBJ, Paxinos G: The Mouse Brain. 3rd ed. Elsevier, Amsterdam, 2008.

Garrett, WT, McBride RL, Williams JK, Feringa ER: Fluoro-Gold's toxicity makes it inferior to True Blue for long-term studies of dorsal root ganglion neurons and motoneurons. Neurosci Lett 128: 137-139 (1991).

Grant G: Neuronal changes central to the axonal transection site. A method for the identification of retrograde changes in perikarya, dendrites and axons by silver impregnation. In: Contemporary Research Methods in Neuroanatomy (Nauta WJH, Ebbesson SOE ed), Springer, Berlin, 1970 (p. 173-185).

Harrison TA, Perry KM, Hoover DB: Regional cardiac ganglia projections in the guinea pig heart studied by postmortem Dil tracing. Anat Rec A Discov Mol Cell Evol Biol 285: 758-770 (2005).

Heimer L: Selective silver-impregnation of degenerating axoplasm. In: Contemporary Research Methods in Neuroanatomy, (Nauta WJH, Ebbesson SOE ed), Springer, Berlin, 1970 (p. 162-172).

Horan PK, Slezak SE: Stable cell membrane labeling. Nature 340: 167-168 (1989).

Ji KH, Xiong J, Fan LX, Hu KM, Liu HQ. Rat marrow-derived multipotent adult progenitor cells differentiate into skin epidermal cells in vivo. J Dermatol 36: 403-409 (2009).

Kassai H, Terashima T, Fukaya M, Nakao K, Sakahara M, Watanabe M, Aiba A: Rac1 in cortical projection neurons is selectively required for midline crossing of commissural axonal formation. Eur J Neurosci 28: 257-267 (2008).)

LaVail JH, LaVail MM. Retrograde axonal transport in the central nervous system. Science 176: 1416-1417 (1972).

Lee EJ, Merwine DK, Mann LB, Grzywacz NM: Ganglion cell densities in normal and dark-reared turtle retinas. Brain Res 1060: 40-46 (2005).

Modo M, Beech JS, Meade TJ, Williams SC, Price J: A chronic 1 year assessment of MRI contrast agent-labelled neural stem cell transplants in stroke. Neuroimage 47: 133-142 (2009).

Nagata I, Terashima T: Migration behavior of granule cells on laminin in cerebellar microexplant cultures from early postnatal reeler mutant mice. Int J Dev Neurosci 12: 387-395 (1994).

O’Leary DD, Terashima T: Cortical axons branch to multiple subcortical targets by interstitial axon budding: implications for target recognition and “waiting periods”. Neuron 1: 901-910 (1988).

Pettersson J, Lobov S, Novikova LN: Labeling of olfactory ensheathing glial cells with fluorescent tracers for neurotransplantation. Brain Res Bull 81: 125-132 (2010).

Secretan C, Bagnall KM, Jomha NM: Effects of introducing cultured human chondrocytes into a human articular cartilage explant model. Cell Tissue Res (in press) (2010).

Sengupta P, Holowka D, Baird B: Fluorescence resonance energy transfer between lipid probes detects nanoscopic heterogeneity in the plasma membrane of live cells. Biophys J 92: 3564-3574 (2007).

Shibata-Iwasaki R, Dekimoto H, Katsuyama Y, Kikkawa S, Terashima T: Anterograde labeling of the corticospinal tract in jimpiny mutant mice by DiI injection into the motor cortex. Arch Histol Cytol 70: 297-301 (2007).
Slezak SE and Horan PK: Fluorescent in vivo tracking of hematopoietic cells. Part 1. *Blood* 74: 2172-2177 (1989).

Smith RL: Axonal projections and connections of the principal sensory trigeminal nucleus in the monkey. *J Comp Neurol* 163: 347-375 (1975).

Stanfield BB: The corticospinal tract attains a normal configuration in the absence of myelin: observations in jimpy mutant mice. *Neuron* 7: 249-256 (1991).

Tang J, Wang J, Kong X, Yang J, Guo L, Zheng F, Zhang L, Huang Y, Wan Y: Vascular endothelial growth factor promotes cardiac stem cell migration via the PI3K/Akt pathway. *Exp Cell Res* 315: 3521-3531 (2009).

Terashima T: Course and collaterals of corticospinal fibers arising from the sensorimotor cortex of the reeler mouse. *Dev Neurosci* 17: 8-19 (1995a).

Terashima T: Anatomy, development and lesion-induced plasticity of rodent corticospinal tract. *Neurosci Res* 22: 139-161 (1995b).

Terashima T, Ochiishi T, Yamauchi T: Alpha calcium/calmodulin-dependent protein kinase II immunoreactivity in corticospinal neurons: combination of axonal transport method and immunofluorescence. *Anat Embryol (Berl)* 192: 123-136 (1995).

Valverde F: The pyramidal tract in rodents. A study of its relations with the posterior column nuclei, dorsolateral reticular formation of the medulla oblongata, and cervical spinal cord. (Golgi and electron microscopic observations). *Z Zellforsch Mikrosk Anat* 71: 298-363 (1966).

Yamagata K, Kumagai K: Experimental study of lymphogenous peritoneal cancer dissemination: migration of fluorescent-labelled tumor cells in a rat model of mesenteric lymph vessel obstruction. *J Exp Clin Cancer Res* 219: 211-217 (2000).

Yamamoto T, Sakakibara S, Mikoshiba K, Terashima T: Ectopic corticospinal tract and corticothalamic tract neurons in the cerebral cortex of yotari and reeler mice. *J Comp Neurol* 461: 61-75 (2003).