Neuron, Vol. 22, 33–41, January, 1999, Copyright ©1999 by Cell Press

A Genetic Approach to Visualization of Multisynaptic Neural Pathways Using Plant Lectin Transgene

Neurotechnique

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Summary

The wiring patterns among various types of neurons via specific synaptic connections are the basis of functional logic employed by the brain for information processing. This study introduces a powerful method of analyzing the neuronal connectivity patterns by delivering a tracer selectively to specific types of neurons while simultaneously transsynaptically labeling their target neurons. We developed a novel genetic approach introducing cDNA for a plant lectin, wheat germ agglutinin (WGA), as a transgene under the control of specific promoter elements. Using this method, we demonstrate three examples of visualization of specific transsynaptic neural pathways: the mouse cerebellar efferent pathways, the mouse olfactory pathways, and the *Drosophila* visual pathways. This strategy should greatly facilitate studies on the anatomical and functional organization of the developing and mature nervous system.

Introduction

Fundamental properties of the nervous system depend critically on intricate but highly ordered patterns of neuronal connections that are constructed and refined during development and maintained and remodeled in adulthood. For the understanding of numerous higher brain functions, including cognitive functions, detailed knowledge of specific and functional neural networks is indispensable.

Plant lectins have been widely used as highly sensitive tracers in anatomical studies for mapping central neural pathways. They are efficiently taken up by neurons and transported in axons and dendrites in both anterograde and retrograde directions. In some cases, injection of lectins in well-mapped central nervous system pathways results in labeling of both first- and second-order neurons and their processes, suggesting that the lectins undergo an interneuronal transfer (Itaya and Van Hoesen, 1982; Shipley, 1985; Baker and Spencer, 1986). Among various lectins, wheat germ agglutinin (WGA) has been extensively studied and proved to be most efficiently transferred between neurons (Broadwell and Balin, 1985; Fabian and Coulter, 1985). In the visual system, for example, the intraocular injection of WGA in monkeys results in labeling of ocular dominance columns in the visual cortex (Itaya and Van Hoesen, 1982; Ruda and Coulter, 1982; Trojanowski, 1983). In the olfactory system, the intranasal administration of WGA in rodents leads to visualization of the primary and secondary olfactory pathways, from the olfactory epithelium to the olfactory bulb and then to the olfactory cortex (Shipley, 1985; Baker and Spencer, 1986; Itaya, 1987). Thus, WGA has been used as an effective marker to delineate transsynaptic circuitry anatomically.

In spite of the above-mentioned usefulness and convenience, there are also limitations and problems in this strategy. First, WGA is taken up by virtually all the neurons located at the injection sites, resulting in nonspecific labeling of unrelated pathways. Thus, the conventional WGA injection method is inappropriate for the visualization of selective and functional neural circuitry originating from a specific type of neuron. Second, WGA is recognized by animals as a foreign substance and causes severe inflammatory and immune responses at the injection site. Third, the conventional WGA method sometimes fails to lead to reproducible results, because of the difficulty in controlling the location and amount of WGA administration.

We have now developed a novel strategy to label specific neural pathways by using WGA cDNA as a transgene. When the WGA transgene was expressed under the control of neuron type–specific promoter elements, anatomically connected and functionally related neural structures were clearly visualized with great accuracy and reproducibility. We have successfully applied this technique to the nervous system of both vertebrate and invertebrate.

Results

WGA Expression in Cultured Neurons

We first examined whether a plant lectin WGA can be efficiently produced and correctly processed to be a mature protein in mammalian cells, especially in neurons, by transfecting WGA cDNA. When a wild-type WGA

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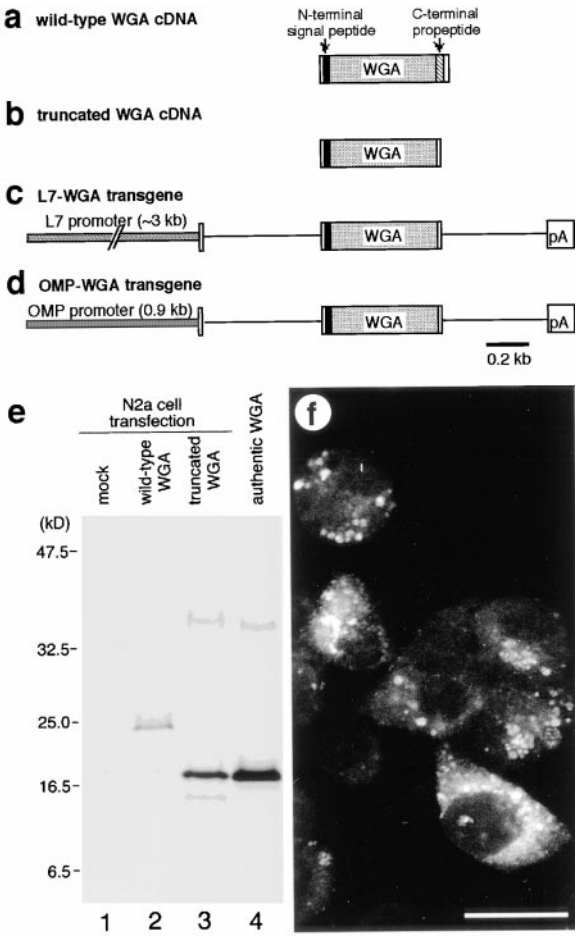

Figure 1. Structural Organization of WGA Transgenes and Expression in N2a Cells

(a) Wild-type WGA cDNA, which encodes a protein with an N-terminal signal peptide (black box) and a C-terminal propeptide (shaded box).

(b) Truncated WGA cDNA which lacks C-terminal propeptide.

(c) L7–WGA transgene containing 3 kb promoter elements of the mouse L7 (Pcp2) gene, truncated WGA cDNA, and SV40 polyadenylation signal (pA).

(d) OMP–WGA transgene containing 0.9 kb promoter elements of the mouse OMP gene, truncated WGA cDNA, and SV40 polyadenylation signal (pA).

(e) Immunoblot analysis of WGA protein. Wild-type WGA expressed in N2a cells migrated as a 24 kDa protein (lane 2), which was larger than the authentic WGA (18 kDa) (lane 4). In contrast, truncated WGA was detected as an appropriately sized 18 kDa band (lane 3) with much more abundance than wild-type WGA.

(f) Immunofluorescent localization of truncated WGA in N2a cells. WGA immunoreactivity was associated strongly with the intracellular granule-like structures. Scale bar, 20 μm.

(Figure 1a) was expressed in mouse neuroblastoma N2a cells, an immunoreactive protein could be detected by Western blot analysis with anti-WGA antibody (Figure 1e, lane 2). However, its molecular size (24 kDa) was significantly larger than that of the authentic WGA (18 kDa) (lane 4). Since the plant WGA has a C-terminal propeptide of 15 amino acids, which is glycosylated, proteolytically cleaved, and implicated in selective delivery of the lectin into vacuoles (Chrispeels and Raikhel, 1991), we speculated that the difference in molecular mass may be due to an absence of such processing machinery in animal cells. Therefore, a truncated WGA that lacks the C-terminal propeptide was constructed by introducing a stop codon at the propeptide cleavage site (Figure 1b). When the truncated WGA was expressed in N2a cells, we detected an appropriately sized band of 18 kDa (Figure 1e, lane 3). In addition, the yield of truncated WGA was much larger than that of wild-type WGA. Immunofluorescence labeling revealed that the truncated WGA protein was associated strongly with the intracellular granule-like structures of N2a cells (Figure 1f), which is reminiscent of the electron microscopical localization of exogenously administered WGA in Golgi-derived vesicles, dense core granules, endosomes, and synaptic vesicles in neurons (Broadwell and Balin, 1985). Hence, we decided to use the truncated WGA as a transgene in all the following experiments.

Cerebellar Efferent Pathways in Mice

L7 (Pcp2) gene promoter has been extensively analyzed (Oberdick et al., 1988, 1990, 1993; Vandaele et al., 1991) and utilized for cerebellar Purkinje cell–specific expression of foreign genes (Feddersen et al., 1992; Burright et al., 1995). We employed the L7 promoter elements (~3.0 kb 5′-flanking region) to direct the expression of WGA in Purkinje cells (Figure 1c) and generated four transgenic mouse lines (L7–WGA 1–4). Southern blot analysis revealed the presence of essentially intact transgenes with copy numbers of 7, 2, 1, and 1 for these lines, respectively (data not shown). In three lines (1, 2, and 4), WGA was expressed robustly by the cerbellar Purkinje cells. The L7–WGA mice were fertile and showed no abnormality in development and behavior.

Localization of WGA mRNA and protein in the brain of L7–WGA 1 transgenic mouse was examined on adjacent parasagittal sections. As expected, strong expression of WGA mRNA was observed exclusively in cerebellar Purkinje cells (Figure 2a). In contrast, WGA protein was detected not only in Purkinje cells but also in several other brain regions that have anatomical and functional relationships with Purkinje cells (Figure 2b). The primary targets of Purkinje cell axons are neurons in the deep cerebellar nuclei, while axons from the flocculonodular lobe project outside the cerebellum to the vestibular nuclei (Ito, 1984; Altman and Bayer, 1996). WGA immunoreactivity was observed in these nuclei with prominent labeling in perikarya of the second-order neurons (Figure 2c). Counterstaining the sections with neutral red revealed that apparently all the deep cerebellar nuclei neurons were positive for WGA (data not shown). Double immunofluorescence labeling with anti-calbindin antibody, a marker for Purkinje cells, confirmed the transsynaptic transfer of WGA protein from the terminals of Purkinje cell axons to the neurons in the deep cerebellar nuclei (Figures 2d–2f).

Output from the cerebellum is mainly by way of the efferent axons of the deep cerebellar nuclei, which are mostly directed toward the mesencephalon and diencephalon. Intense WGA labeling was detected in the red nucleus and the thalamic ventrolateral nucleus, two major targets of the cerebellar efferent axons (Figures 2g–2j). Furthermore, WGA immunoreactivity was detected in several other structures to which the cerebellar

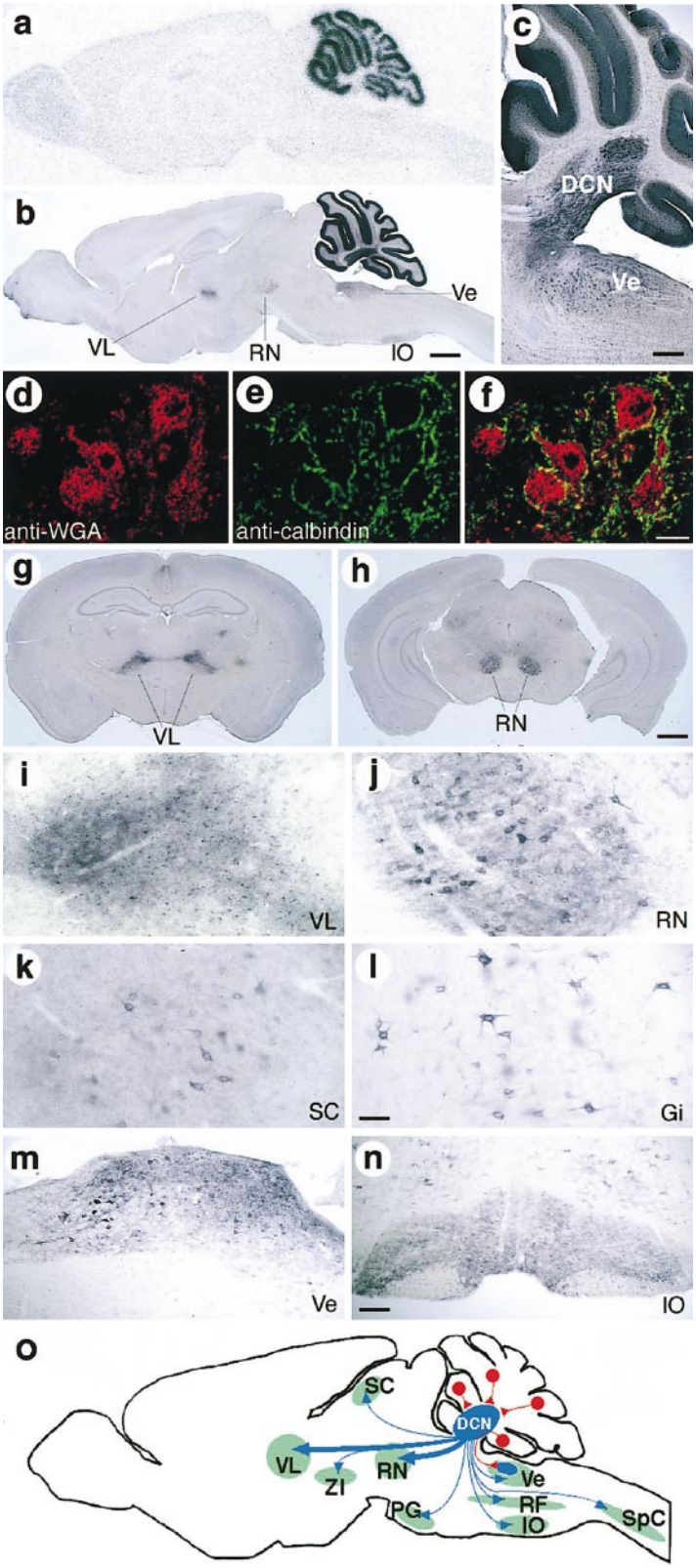

Figure 2. Visualization of Cerebellar Efferent Pathways in L7–WGA Transgenic Mice

(a) In situ hybridization of a parasagittal section of the L7–WGA mouse brain revealed that WGA mRNA expression is confined to the cerebellar Purkinje cells.

(b) Immunohistochemistry of an adjacent section revealed that WGA protein was detectable not only in the cerebellar Purkinje cells but also in the deep cerebellar nuclear neurons, and even in several nuclei outside the cerebellum such as the thalamic ventrolateral nucleus (VL), the red nucleus (RN), the vestibular nucleus (Ve), and the inferior olive (IO).

(c) WGA was detected in the second-order neurons in three subnuclei of the deep cerebellar nuclei (DCN) as well as in the vestibular nuclei (Ve).

(d–f) Double immunofluorescence labeling of the second-order neurons in the deep cerebellar nuclei with anti-WGA (d and f) and anti-calbindin (e and f) antibodies. Calbindin was present only in axon terminals of the Purkinje cells, whereas WGA was detectable also in the intracellular granule-like structures of the second-order neurons.

(g–j) The thalamic ventrolateral nucleus (VL in [g] and [i]) and the red nucleus (RN in [h] and [j]), both of which recieved massive axonal projections from the deep cerebellar nuclei, were strongly positive for WGA. High-power views (i and j) showed WGA uptake into the third-order neurons.

(k and l) Other target regions of the cerebellar efferents such as the superior colliculus (SC in [k]) and the gigantocellular reticular nucleus (Gi in [l]) were also WGA positive.

(m) The vestibular nucleus (Ve), which received both mono- and di-synaptic inputs from the Purkinje cells, was strongly WGA positive.

(n) The inferior olivary nucleus (IO) contained WGA immunoreactivity. This labeling may have been derived from the retrograde WGA transport from the Purkinje cells through the climbing fibers and/or the anterograde transport from the deep cerebellar nuclei and the red nucleus.

(o) Schematic diagram of the cerebellar efferent pathways. The first-order neurons (Purkinje cells) are shown in red. The second-order neurons in DCN (blue) project their axons to several target nuclei (green), all of which were labeled in L7–WGA mice. Other abbreviations: PG, pontine gray nucleus; RF, reticular formation; SpC, spinal cord; ZI, zona incerta.

Scale bars: 1 mm (a and b), 300 μm (c), 20 μm (d–f), 1 mm (g and h), 60 μm (i–l), and 120 μm (m and n).

efferent axons project, such as the superior colliculus, the reticular formation, the vestibular nucleus, and the inferior olive (Figures 2k–2n). In all these regions, surprisingly, WGA uptake was observed in the third-order neurons (Figures 2i–2n). These results clearly establish multisynaptic transfer of the WGA protein along a specific neuronal pathway. WGA protein produced by Purkinje cells is transported via their axons to second-order neurons in deep cerebellar nuclei, and thereafter further transported through axons of cerebellar nuclear neurons

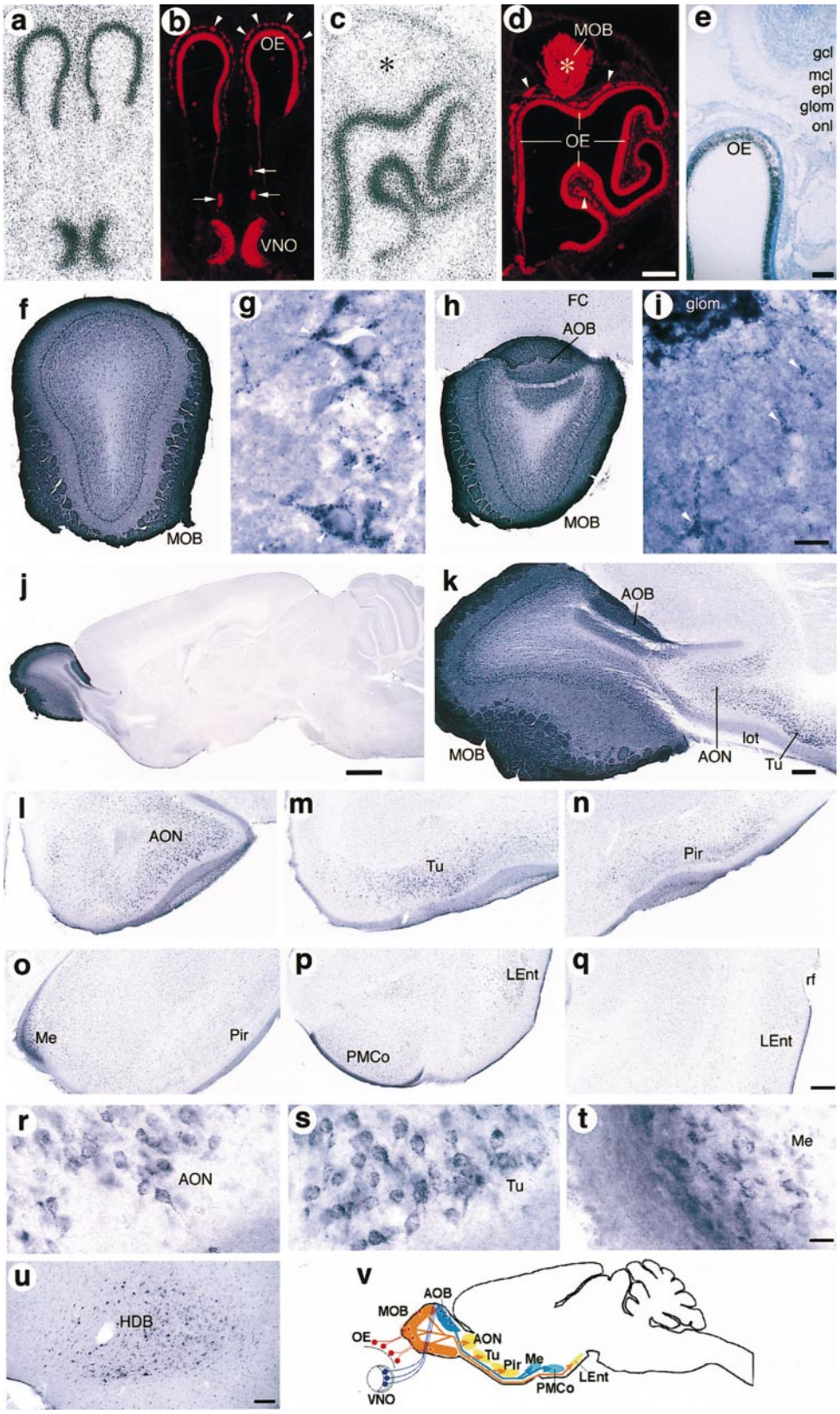

Figure 3. Visualization of Olfactory Pathways in OMP–WGA Transgenic Mice

(a and b) In situ hybridization (a) and immunohistochemistry (b) of adjacent coronal sections at the rostral level of OMP–WGA mouse nose. WGA mRNA and protein were robustly expressed in both the olfactory epithelium (OE) and the vomeronasal organ (VNO). WGA protein was present also in the axonal bundles originating from OE (arrowheads) and from VNO (arrows).

to third-order neurons in mesencephalon and diencephalon (Figure 2o). A similar labeling pattern of the cerebellar efferent pathways was observed also in the other two lines, 2 and 4, of L7–WGA mice (data not shown).

Olfactory Pathways in Mice

Olfactory marker protein (OMP), a 19 kDa cytosolic protein with unknown function, is selectively and abundantly expressed by mature olfactory and vomeronasal sensory neurons (Margolis, 1985, 1993; Danciger et al., 1989; Buiakova et al., 1994). The *cis*-acting elements of OMP gene have been successfully utilized for expression of foreign genes in these sensory neurons (Largent et al., 1993; Holtmaat et al., 1995; Walters et al., 1996). We generated four mouse lines (OMP–WGA 1–4) that carried the integrated hybrid OMP promoter (0.9 kb)–WGA transgene (Figure 1d). Southern analysis revealed the presence of transgenes with copy numbers of 8, 5, 1, and 30 for these lines, respectively (data not shown). The OMP–WGA mice displayed normal development and behavior, except for the line 1 mice, which showed marked reduction of the offspring number for some reason.

Figure 3 shows the result of the OMP–WGA line 2, as a representative transgenic line in which both the olfactory and vomeronasal pathways could be clearly visualized. In situ hybridization analysis revealed that WGA mRNA was expressed exclusively in all the mature sensory neurons in both the olfactory and vomeronasal epithelia (Figures 3a, 3c, and 3e), faithfully to the endogenous OMP expression (Danciger et al., 1989; Buiakova et al., 1994). In contrast, no mRNA signal could be detected in cells in the brain (Figures 3c [asterisk] and 3e). Adjacent sections subjected to WGA immunohistochemistry showed the presence of WGA protein in soma of these sensory neurons and in axon bundles leaving from the olfactory epithelium (arrowheads in Figures 3b and 3d) and from the vomeronasal epithelium (arrows in Figure 3b). In the main and accessory olfactory bulbs, WGA immunoreactivity was detected not only in the axon terminals in glomeruli but also in the external plexiform layer, the mitral cell layer, and the granule cell layer (Figures 3f–3k). A higher magnification revealed that perikarya of the mitral/tufted cells, the second-order neurons of the olfactory pathways, were intensely WGA positive (Figures 3g and 3i), indicating that the WGA protein underwent the transsynaptic transfer also in this system. Counterstaining the sections with neutral red confirmed the WGA uptake in all the mitral/tufted cells (data not shown).

The presence of intense label in mitral/tufted cell perikarya suggested that the WGA protein might be further transported to the olfactory cortex. The WGA immunoreactivity was observed in the four major terminal fields of the mitral/tufted cell axons of the main olfactory system; the anterior olfactory nucleus (Figures 3k, 3l, and 3r), the olfactory tubercle (Figures 3k, 3m, and 3s), the piriform cortex (Figures 3n and 3o), and the lateral entorhinal cortex (Figures 3p and 3q) (Shipley et al., 1995). In addition, two major projection sites from the accessory olfactory bulb were also strongly positive for WGA; the medial amygdaloid nucleus (Figures 3o and 3t) and the posteromedial cortical amygdaloid nucleus (Figure 3p) (Shipley et al., 1995). Many neurons in these areas of the olfactory cortex contained WGA-positive granule-like profiles, suggesting the transsynaptic labeling of the third-order neurons (Figures 3r–3t). Figure 3u shows the retrograde transsynaptic labeling of neurons in the horizonatal limb of the diagonal band, which send massive projections to the main olfactory bulb (Shipley et al., 1995). A schematic diagram of the anterograde transsynaptic labeling of the olfactory pathways is summarized in Figure 3v.

(c and d) In situ hybridization (c) and immunohistochemistry (d) of adjacent coronal sections at the more caudal level of the nose. WGA mRNA expression was confined to OE, whereas WGA protein was transported via olfactory axons (arrowheads) to the main olfactory bulb (MOB; asterisk in [d]). Note the absence of WGA mRNA in MOB (asterisk in [c]).

(e) A high-power bright-field micrograph of WGA mRNA expression. Again, note the strong WGA mRNA expression in OE, but not in MOB. Other abbreviations: gcl, granule cell layer; mcl, mitral cell layer; epl, external plexiform layer; glom, glomerular layer; onl, olfactory nerve layer.

(f) A coronal section showing WGA protein in MOB. The olfactory axons and their terminals in glomeruli were heavily labeled. In addition, the mitral cells were strongly positive for WGA immunoreactivity, and the external plexiform and granule cell layers were also moderately immunopositive.

(g) A high-power view of the mitral cells in MOB. WGA signals were found in the intracellular granule-like structures in the mitral cells (arrowheads).

(h) A coronal section showing WGA protein in the accessory olfactory bulb (AOB) and MOB. Note the absence of WGA immunoreactivity in the frontal cortex (FC).

(i) A high-power view of AOB showing WGA transfer to the mitral/tufted cells (arrowheads).

(j) A low-power parasagittal view showing the specific WGA labeling in the olfactory system.

(k) A parasagittal view showing that WGA transferred to the mitral/tufted cells is further transported to olfactory cortical areas such as the anterior olfactory nucleus (AON) and olfactory tubercle (Tu) via the lateral olfactory tract (lot).

(l–q) Major projection sites of the mitral/tufted cell axons. The mitral/tufted cell axons of MOB projected to various cortical structures, including the anterior olfactory nucleus (AON in [l]), the olfactory tubercle (Tu in [m]), the piriform cortex (Pir in [n] and [o]), and the lateral entorhinal cortex (LEnt in [p] and [q]), whereas those of AOB projected to two major sites, the medial amygdaloid nucleus (Me in [o]) and the posteromedial cortical amygdaloid nucleus (PMCo in [p]). Other abbreviation: rf, rhinal fissure.

(r–t) High-power micrographs of AON (r), Tu (s), and Me (t) showing the WGA transfer to the third-order neurons.

(u) Retrograde WGA transfer from the olfactory bulb to the neurons in the horizontal limb of the diagonal band (HDB).

(v) Schematic diagram of the olfactory pathway. In the main olfactory pathway, the first-order neurons (olfactory sensory neurons, red) produce and transfer WGA protein to the second-order neurons in MOB (orange), which project their axons to the four major target regions (yellow). In the accessory olfactory pathway, the first-order neurons (vomeronasal sensory neurons, dark blue) produce and transfer WGA protein to the second-order neurons in AOB (blue), which project their axons to the two major target regions (light blue).

Scale bars:, 500 μm (a–d), 100 μm (e), 200 μm (f, h, and k), 10 μm (g and i), 1 mm (j), 200 μm (l–q), 20 μm (r–t), and 100 μm (u).

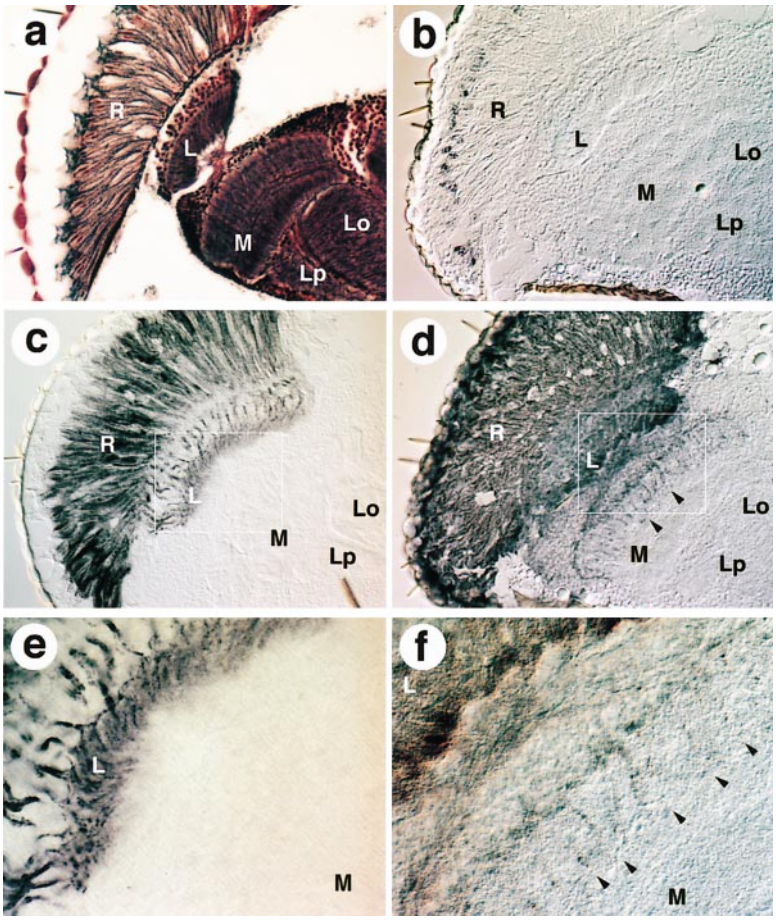

Figure 4. WGA Transgene Expression in *Drosophila* Visual System

Horizontal sections of the adult *Drosophila* head.

(a) A silver-stained section of the wild-type retina (R) and optic lobe. *Drosophila* optic lobe consists of the four neuropil layers: the lamina (L), the medulla (M), the lobula (Lo), and the lobula plate (Lp).

(b) GAL4 immunoreactivity of *Rh1–GAL4; UAS–WGA*. GAL4 was detected only in the nuclei of R1–R6 cells in the retina.

(c) Tau immunoreactivity of *Rh1–GAL4; UAS–Tau*. Tau-positive axons of outer photoreceptors projected to the lamina.

(d) WGA immunoreactivity of *Rh1–GAL4; UAS–WGA* (adjacent section of [b]). In addition to intense signal in the retina and lamina, WGA was detected in the axons of the second-order neurons (arrowheads).

(e) A high-power view of (c) (inside of white frame). Tau-positive axons terminated in the lamina, and there was no signal in the medulla.

(f) A high-power view of (d) (inside of white frame). WGA immunoreactivity was present in the axonal terminals of the second-order neurons (arrowheads).

A similar labeling pattern of the olfactory and vomeronasal pathways was observed also in another line (4) of OMP–WGA mice (data not shown). In contrast, WGA transgene expression was confined to subsets of OMP-expressing sensory neurons in line 1 (data not shown), probably due to the influence of the genomic insertion site of the transgene (Jaenisch, 1988). In line 3, WGA expression could not be detected in any tissues.

Visual Pathways in *Drosophila*

Next, a cell type–specific expression of the WGA transgene was driven in the adult *Drosophila* visual system by using the GAL4/UAS system (Brand and Perrimon, 1993). An *Rh1–GAL4* line (Hama and Sone, unpublished data), in which GAL4 transcription is directed by the enhancer/promoter of the *Rh1* gene, was used to drive the expression of transgenes specifically in R1–R6 photoreceptor cells in the retina (Mismer and Rubin, 1987; Masai et al., 1997). Axons of the R1–R6 cells project to a neuropil, called the lamina, where they synapse with the second-order neurons (monopolar cells). The second-order neurons then project to another neuropil, the medulla (Figure 4a) (Meinertzhagen and Hanson, 1993). GAL4 immunoreactivity in the transgenic flies was restricted to the nuclei of R1–R6 photoreceptor cells (Figure 4b). Horizontal sections of adult heads from two transgenic lines (*Rh1–GAL4; UAS–Tau* and *Rh1–GAL4; UAS–WGA*) were stained with anti-Tau and anti-WGA antibodies, respectively. Expression of Tau was restricted to the retina and lamina, corresponding to cell bodies and axons of the first-order neurons (Figures 4c and 4d) (Ito et al., 1997). In contrast, a striped pattern of anti-WGA labeling was observed in the outer half of the medulla to which axons of the second-order neurons project, in addition to the stronger labeling found in the retina and lamina (Figures 4e and 4f). These results suggest that the WGA protein was transsynaptically transferred from the R1–R6 cells to the second-order neurons. Thus, the WGA transgene technique will be useful for transsynaptic tracing not only in mouse but also in *Drosophila*, and perhaps in other species. The availability of the *UAS–WGA* line will definitely facilitate the elucidation of a number of neuronal circuits in *Drosophila*, as it can be used with any neurally expressing *GAL4* lines.

Discussion

We have presented a novel approach for visualizing specific neural circuitry using WGA as a transgene. In two types of transgenic mice and one type of transgenic *Drosophila*, WGA was produced under the tight control of cell type–specific promoters. WGA protein produced in the first-order neurons was efficiently transported anterogradely and then transsynaptically transferred to the

second-order neurons. In some cases, surprisingly, we observed the WGA uptake into the third-order neurons.

Features of WGA Transgene

A wild-type WGA has an N-terminal 27 amino acid signal peptide for the entry into endoplasmic reticulum and a C-terminal 15 amino acid propeptide for the following delivery into vacuoles in wheat germ (Mansfield et al., 1988; Chrispeels and Raikhel, 1991). Using the cell culture system, we first asked whether the plant lectin can be efficiently produced and properly processed in animal cells. From the result of Western blot analysis (Figure 1e), it was evident that the C-terminal propeptide of a wild-type WGA was unnecessary and rather obstructive, probably due to different processing and sorting mechanisms between plant and animal cells. Judging from the molecular mass and yield of the product, we concluded that a truncated WGA lacking the propeptide is suitable for expression in animal cells.

WGA protein was localized to the intracellular granule-like structures in cultured N2a cells and brain neurons. Broadwell and Balin (1985) reported that the in vivo administered WGA undergoes endocytotic and exocytotic intracellular pathways of the neuronal secretory process and transsynaptic transfer. By using electron microscopy, they showed that WGA occurs in granule-like structures in neurons, such as endosomes, primary lysosomes, multivesicular bodies, *trans*-Golgi saccules, dense core secretory granules, and synaptic vesicles. Similar appearance of WGA in neurons in the present study suggests that, following the synthesis and maturation in endoplasmic reticulum and Golgi network, the WGA transgene product enters the same endocytotic and secretory pathways.

WGA produced in the first-order neurons was transported both anterogradely into axons and retrogradely into dendrites. The anterogradely transported WGA in the axonal terminals of the first-order neurons underwent the transsynaptic transfer to the second-order neurons. Then, is there any retrograde transsynaptic transfer of WGA from postsynaptic dendrites to presynaptic axons? In all the lines of L7–WGA mice, weak but significant WGA immunoreactivity was detected in the cerebellar granule cells (Figure 2c), whose axons (parallel fibers) make synaptic contacts with Purkinje cell dendrites. In the OMP–WGA mice, furthermore, strong WGA immunoreactivity was observed in neurons in the nucleus of the diagonal band (Figure 3u), which massively project centrifugal axons (cholinergic and GABAergic) to the main olfactory bulb (Shipley et al., 1995). These results demonstrate occurrence of the retrograde transsynaptic transfer of WGA from dendrites to axons.

With the conventional WGA administration method for transsynaptic labeling, there were several limitations and problems such as impossiblitity of cell type–specific expression, severe immune response by foreign WGA, and difficulty in reproducibility of the experiments. Our strategy, described here, overcame these problems. First, we succeeded in forced expression of WGA in a very restricted subset of neurons. Second, since WGA was produced as an endogenous molecule in transgenic animals, it caused no inflammatory or immune responses. Third, we obtained essentially the same results with animals of the same line with great reproducibility and accuracy.

Wider Applications of WGA Transgene

Visualization of specific multisynaptic neural pathways is indispensable for understanding brain functions, especially higher cognitive functions that involve combinations of intricate neural networks widely distributed in the brain. The technique described here permitted us to deliver plant lectin WGA to specific types of neurons and selectively label transsynaptic neural pathways at least to second-order neurons and usually to third-order neurons that resided in regions far apart from the first-order neurons. Thus, this technique is applicable to a variety of neurobiological studies on the development, anatomy, and functions of the brain. For example, if the beginning of transsynaptic transfer of WGA from the first- to the second-order neurons precisely correlates with the timing of synapse formation between these neurons, the development of functional synapses can be analyzed by monitoring the WGA appearance in the second-order neurons. Another extension of the WGA transgenic mice is visualization of changes of specific neural pathways in various spontaneous and gene-targeted mutant mice. This can be accomplished by simply mating WGA transgenic mice and mutant mice and investigating specific neural pathways (e.g., the cerebellar efferent and the olfactory pathways) by WGA immunohistochemistry in comparison with wild-type animals.

However, there may be limitations in the WGA transgene technique. WGA transfer across synapses seems to depend on the expression levels of WGA protein in the first-order neurons. Here, we employed very strong promoter elements (L7 and OMP) that direct robust and sufficient expression of WGA protein for visualization of multisynaptic neural pathways. A problem of insufficient transsynaptic labeling might occur in instances when the expression levels of WGA are much lower due to the weakness of certain promoter activities. This problem will be circumvented by use of the conditional WGA expression with a strong promoter element linked to a Cre/*loxP* recombination system. We are now making transgenic mice with the following construct: ubiquitous and strong promoter–*loxP*–stop signal–*loxP*–WGA cDNA. By mating these mice with Cre-expressing transgenic mice (neuron type–specific but weak promoter–Cre), we expect the high levels of WGA production exclusively in Cre-expressing neurons in which the stop signal is excised by the Cre-mediated DNA recombination. In such mice, weak promoter elements could be used for the transsynaptic labeling of specific neural pathways with WGA transgene. Another limitation may be encountered when the promoters drive the WGA expression in more discrete subsets of neurons in several brain regions. This problem may be overcome by use of the adenoviral vector system, which enables us to localize the WGA expression in a certain brain region. The adenoviral vectors containing WGA cDNA downstream of neuron type–specific promoter elements will be used for the restricted expression of WGA in time-, place-, and cell type–specific manners.

Further refinement of the technique is to use a chimeric molecule consisting of WGA and green fluorescent

protein (GFP) as a transsynaptic fluorescent reporter. If WGA–GFP can be efficiently transferred across synapses, specific neural pathways will be visualized in vivo simply by monitoring the GFP fluorescence. Such improvement of the technique will further extend the application of WGA transgene. Thus, the present technique for visualization of specific multisynaptic neural pathways will provide an extremely valuable tool for the studies of formation, refinement, maintenance, and remodeling of neural networks in the brain.

Experimental Procedures

Plasmid Construction

A wild-type WGA cDNA insert (1.0 kb) excised from pWGA–D (Smith and Raikhel, 1989) was blunt ended, BstXI adapted, and subcloned into a BstXI site of a mammalian expression vector pEF–BOS (Mizushima and Nagata, 1990), generating plasmid pEF–WGA. pEF–tWGA containing a C terminus–truncated WGA cDNA was generated from pEF–WGA by replacing a codon GTC (valine 198) with a stop codon TGA using PCR-based mutagenesis. This mutation results in removal of a C-terminal propeptide (15 amino acid residues).

To construct pL7–tWGA, a mouse L7 promoter region (3.5 kb) was PCR amplified from Pcp2–Z06 plasmid (Vandaele et al., 1991) and subcloned into a blunt-ended BamHI site of pBstN vector, which contains human β-globin gene introns and an SV40 polyadenylation signal. tWGA cDNA sequence (0.6 kb) excised from pEF–tWGA was ligated into a blunt-ended EcoRI site of pBstN with L7 promoter.

To create pOMP–tWGA, an OMP promoter region (0.9 kb) was PCR amplified from mouse genomic DNA and subcloned into a blunt-ended BamHI site of pBstN vector. tWGA cDNA sequence was inserted as described above.

The pUAST–WGA plasmid was constructed by inserting the 0.6 kb XbaI fragment of pEF–tWGA into the XbaI site of the pUAST vector (Brand and Perrimon, 1993).

N2a Cell Transfection

Mouse neuroblastoma N2a cells were transfected with plasmid DNA using Lipofectamine and Opti-MEM (Gibco/BRL). After 48 hr, the cells were used for Western blot analysis and immunofluorescence labeling with anti-WGA polyclonal antibody (10 μg/ml, Sigma). The authentic WGA used as a standard for Western analysis was purchased from Sigma. The labeled cells were examined with a confocal laser scanning microscopy system (Bio-Rad MRC-600) equipped with a Zeiss Axiophot F1 microscope.

Generation of Transgenic Mice

Transgenic mouse lines were generated by microinjection of fertilized eggs as described (Nohmi et al., 1996). The purified inserts of pL7–tWGA and pOMP–tWGA were injected into the male pronucleus of fertilized eggs from the FVB/N (CLEA Japan) strain of mice. The manipulated eggs were cultured and transferred into the oviducts of ICR (CREA Japan) pseudopregnant recipients. Integration of the transgenes was screened by PCR and Southern blot analysis of tail DNA.

Generation of Transgenic *Drosophila*

The pUAST–WGA plasmid was injected into *w^{1118}; Dr/TMS, Sb P[ry^{+}, Æ2-3]* embryos as previously described (Sawamoto et al., 1994). Seven independent transformant lines were obtained. The *Rh1–GAL4* line was a gift from Dr. C. Hama (National Institute for Neuroscience, Japan). The *UAS–Tau GFP 5.8* line (Brand, 1995) was a gift from Dr. A. H. Brand (Cambridge University, United Kingdom).

Immunohistochemistry

Sections (50 μm) of paraformaldehyde-perfused mouse brains were cut with a sliding microtome, pretreated with 0.3% H_2O_2, blocked, and incubated for 2–24 hr at room temperature with anti-WGA polyclonal antibody (3 μg/ml, Sigma) that had been preabsorbed with 1% acetone powder of mouse brains. The sections were then incubated with either biotin anti-rabbit IgG (Zymed) followed by a Vectastain ABC elite kit (Vector) or horseradish peroxidase anti-rabbit IgG (Jackson). Signals were visualized with the Ni^{2+}-intensified diaminobenzidine/peroxide reaction. In the double immunofluorescence labeling, Cy3 anti-rabbit IgG (Jackson) and FITC anti-mouse IgG (Cappel) were used as the second antibodies, and the labeled sections were analyzed with a confocal laser scanning microscopy system. A monoclonal anti-calbindin D28K antibody was purchased from Sigma.

Adult fly heads were removed by dissection and embedded in Tissue Tek OCT compound (Miles). Frozen sections (9 μm) were fixed immediately in 4% paraformaldehyde in PBS for 30 min and stained with anti-WGA antibody (Sigma) preabsorbed with 1% acetone powder of *Drosophila* head, anti-Tau monoclonal antibody (Sigma), or anti-GAL4 monoclonal antibody (Clontech) as described above. Silver staining of *Drosophila* was performed as previously described (Meyerowitz and Kankel, 1978).

The preabsorption procedure of anti-WGA antibody was necessary for accurate detection of the WGA transgene product. No immunopositive signal was detected in nontransgenic mice and *Drosophila* with the preabsorbed anti-WGA antibody, whereas the original antibody cross-reacted with unknown endogenous molecule(s) (data not shown).

In Situ Hybridization

All steps of in situ hybridization were performed essentially as described (Yoshihara et al., 1997). Sections (20 or 50 μm) of paraformaldehyde-fixed mouse tissues were treated with proteinase K (10 μg/ml at 25°C for 30 min), acetylated, dehydrated, and air dried. An antisense riboprobe for WGA (540 nucleotides in length) was prepared with [^{35}S]UTP (Amersham) and RNA transcription kit (Stratagene). The sections were hybridized overnight at 56°C in a humidified chamber with 1×10^6 cpm/ml ^{35}S-labeled cRNA probe. After hybridization, the sections were washed in 4× SSC, treated with RNase A (10 μg/ml at 37°C for 30 min), washed in 0.05× SSC, dehydrated with ethanol, and exposed to βmax x-ray film (Amersham). After autoradiography, slides were dipped in NTB-2 emulsion, exposed, developed in Kodak D-19, fixed with RenFix, and counterstained with cresyl violet.

Acknowledgments

The authors are grateful to Richard Axel for generous support and critical comments on the manuscript. We also thank Shigetada Nakanishi, Fujio Murakami, and Kei Ito for valuable discussion; Natasha V. Reikhel and Yoshifumi Jigami for WGA cDNAs; Harry T. Orr for Pcp2 (L7) promoter; Frank L. Margolis for OMP promoter; and Chihiro Hama and Masaki Sone for *Rh1–GAL4 Drosophila* line. This work was supported in part by the Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

Received August 31, 1998; revised December 1, 1998.

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