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Adenovirus-mediated WGA Gene Delivery for Transsynaptic Labeling of Mouse Olfactory Pathways

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Abstract

Detailed knowledge of neuronal connectivity patterns is indispensable for studies of various aspects of brain functions. We previously established a genetic strategy for visualization of multisynaptic neural pathways by expressing wheat germ agglutinin (WGA) transgene under the control of neuron type-specific promoter elements in transgenic mice and *Drosophila*. In this paper, we have developed a WGA-expressing recombinant adenoviral vector system and applied it for analysis of the olfactory system. When the WGA-expressing adenovirus was infused into a mouse nostril, various types of cells throughout the olfactory epithelium were infected and expressed WGA protein robustly. WGA transgene products in the olfactory sensory neurons were anterogradely transported along their axons to the olfactory bulb and transsynaptically transferred in glomeruli to dendrites of the second-order neurons, mitral and tufted cells. WGA protein was further conveyed via the lateral olfactory tract to the olfactory cortical areas including the anterior olfactory nucleus, olfactory tubercle, piriform cortex and lateral entorhinal cortex. In addition, transsynaptic retrograde labeling was observed in cholinergic neurons in the horizontal limb of diagonal band, serotonergic neurons in the median raphe nucleus, and noradrenergic neurons in the locus coeruleus, all of which project centrifugal fibers to the olfactory bulb. Thus, the WGA-expressing adenovirus is a useful and powerful tool for tracing neural pathways and could be used in animals that are not amenable to the transgenic technology.

Introduction

Information transfer between neurons takes place at the synapse. 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. Accordingly, detailed knowledge on neuronal connectivity patterns is essential for understanding the wide range of brain functions.

Plant lectins have been used as highly sensitive tracers in neuroanatomical 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 neural pathways results in labeling of both first- and second-order neurons and their processes, suggesting that the lectins undergo an interneuronal transfer. Among various lectins, wheat germ agglutinin (WGA) has been studied extensively and proved to be transferred most efficiently between neurons (Broadwell and Balin, 1985; Fabian and Coulter, 1985). In the visual system, for example, the intraocular injection of WGA protein 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 rodent olfactory system, the intranasal administration of WGA protein leads to visualization of the primary and secondary olfactory pathways, from the olfactory epithelium to the bulb and then to the cortex (Shipley, 1985; Baker and Spencer, 1986; Itaya, 1987).

We have recently developed a novel genetic strategy for visualization of selective neural pathways across synapses by combining a neuroanatomical tracing method with a transgenic technology (Yoshihara *et al.*, 1999; Tabuchi *et al.*, 2000). By introducing cDNA encoding WGA, as a transgene under the control of specific promoter elements, selective and functional transsynaptic neural pathways could be visualized. For example, when WGA transgene was expressed specifically in Purkinje cells in the mouse cerebellum under the control of L7 promoter, WGA protein produced by the Purkinje cells (first-order neurons) was transported through their axons to nerve terminals and transferred across a synapse to the second-order neurons in the deep cerebellar nuclei. Furthermore, WGA was conveyed to the third-order neurons in the midbrain red nucleus and the thalamic ventrolateral nucleus, permitting us to track the cerebellar efferent pathways. Similarly, we have succeeded in

visualization of mouse olfactory pathways and *Drosophila* visual pathways. Thus, the WGA transgene technology provides an extremely valuable tool for the studies of formation, maintenance and remodeling of neural networks in the brain.

Viral vectors are becoming increasingly important tools for foreign gene expression in various studies of neuroscience. In contrast to the transgenic mouse system, viral vectors can be applied to transfer genes in time- and place-specific manners in fully developed animals. In this paper, the WGA transgene technique was modified, combining it with the recombinant adenoviral vector system, which was successfully applied to visualizing the mouse olfactory system. This new technique makes the process more convenient and potentially opens it up to wider applications.

Materials and methods

Cells

HEK293 human embryonic kidney cells and N2a mouse neuroblastoma cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Viral preparation

Recombinant adenovirus for expression of WGA was prepared according to the method using a cassette cosmid pAxCAwt (Miyake *et al.*, 1996). Briefly, truncated WGA cDNA which encodes WGA protein without carboxy-terminal propeptide (Yoshihara *et al.*, 1999) was blunt-ended and inserted into pAxCAwt at the SwaI site. In this way, the inserted cDNA is transcribed under the control of a powerful and ubiquitous CAG promoter which harbors the cytomegalovirus immediate early enhancer and chicken β-actin promoter (Niwa *et al.*, 1991). The cosmid DNA was cotransfected with the EcoT22I-digested DNA-terminal protein complex of Ad5-DlX into HEK293 cells to generate the recombinant virus by homologous recombination. The recombinant virus, designated AxCAWGA, was propagated in HEK 293 cells. After the fourth propagation, virions were extracted, purified by double CsCl step-gradient centrifugation (Kanegae *et al.*, 1994), dialyzed against a vehicle solution containing 10% glycerol in PBS, pH 7.4, and stored at –80°C. The titer of recombinant virus was determined by the modified end-point cytopathic effect assay on HEK293 cells (Kanegae *et al.*, 1994) and expressed in plaque-forming units (pfu) per ml. Positive expression of the inserted gene product was confirmed by Western blot analysis and immunocytochemical detection using mouse neuroblastoma N2a cells. Experiments using recombinant adenovirus were approved by the Recombinant DNA Committee of RIKEN and performed according to institutional guidelines.

Viral infection

For cultured cell infection, AxCAWGA (3×10^8 pfu/ml, 2 μl) was added to N2a cells plated onto either six-well plates (5×10^5 cells/well) or glass chamber slides (1×10^5 cells/well) (Becton Dickinson, Franklin Lakes, NJ). After 48 h, the cells were analyzed for WGA protein expression by Western blotting and immunocytochemistry using anti-WGA antibody (Sigma, St Louis, MO).

For *in vivo* infection, 4- to 12-week-old male ddY mice (Nihon SLC, Hamamatsu, Japan) were used. Animals were anesthetized with pentobarbital (2.5 mg/mouse, i.p.). A drop (~0.5 μl) of PBS solution containing the virus AxCAWGA at a titer of $2–5 \times 10^9$ pfu/ml was put at the entrance of right nasal cavity. After all the solution entered into the cavity by animal's spontaneous respiration, another drop was applied. This process was repeated >100 times over a period of ~60 min, until the total volume of applied virus solution reached 50–70 μl. This simple method enabled us to infect the virus throughout the nasal epithelium almost evenly. After 7, 12, 20, 30 and 50 days of survival, the mice were sacrificed for analysis of WGA expression.

WGA immunohistochemistry

Paraformaldehyde-perfused mouse nasal epithelia or brains were cut with a cryostat or a sliding microtome to obtain 20 or 50 μm sections, respectively. The sections were pretreated for 30 min with 0.3% H_2O_2 in PBS to inactivate endogenous peroxidase activity and incubated for 30 min with PBS containing 0.2% Triton X-100 and 5% normal goat serum (PBST/NGS) for permeabilization of cells and blocking of nonspecific protein-binding sites. The sections were then incubated for 2 h with anti-WGA polyclonal antibody (3 μg/ml, Sigma) in PBST/NGS that had been pre-absorbed with 1% acetone powder of mouse brains. This pre-absorption procedure of anti-WGA antibody was necessary for accurate detection of the WGA transgene product, because the non-absorbed antibody cross-reacted with unknown endogenous molecule(s) in the mouse brain. The sections were then incubated with biotin-labeled anti-rabbit IgG (Jackson, West Grove, PA) followed by Vectastain ABC elite kit (Vector, Burlingame, CA). Signals were visualized with Ni^{2+}-intensified diaminobenzidine/peroxide reaction. Specimens were observed by using a light microscope equipped with differential interference contrast optics (Olympus, Tokyo, Japan: Provis), a cooled CCD camera (Sony, Tokyo, Japan), and an image analysis system (Adobe Photoshop, San Jose, CA). For immunofluorescence labeling, Cy3-conjugated anti-rabbit IgG (Jackson) was used as the second antibody. The labeled sections were analyzed with a confocal laser scanning microscopy system (Leica TCS SP, Heidelberg, Germany).

Evaluation of WGA immunoreactivity levels in various regions of interest was performed as a blind experiment by comparing the stained sections of all mice perfused at different time points in accordance with following criteria: very high (+++), high (++), weak but significant (+), faint (±), or no expression (–) of the WGA transgene product.

Results

Adenovirus-mediated WGA expression in cultured neurons

A recombinant adenoviral vector for WGA expression under the control of strong and ubiquitous CAG promoter elements was constructed, purified and concentrated. We first examined whether this virus can efficiently mediate a high level of WGA expression in cultured cells. When the recombinant virus AxCAWGA was infected into mouse neuroblastoma N2a cells, an intense immunoreactive band was detected by Western blot analysis, whose molecular size (~18 kDa) corresponded to that of authentic WGA protein (Figure 1A). Immunofluorescence labeling revealed that WGA protein was associated strongly with the intracellular granule-like structures of N2a cells (Figure 1B), 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). Thus, it was confirmed that adenovirus-mediated WGA expression occurs efficiently in cultured neurons.

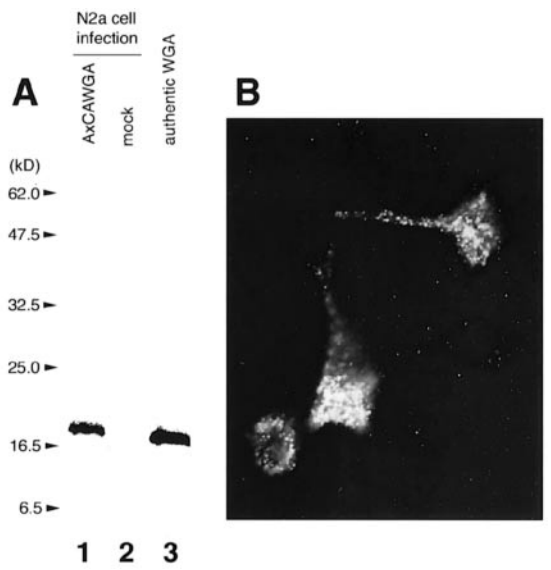

Figure 1 Adenovirus-mediated WGA expression in N2a cells. **(A)** Immunoblot analysis. Mouse neuroblastoma N2a cells were infected with AxCAWGA (lane 1) or mock-infected (lane 2). After 48 h of infection, the cells were lysed and subjected to SDS–PAGE followed by Western blot analysis with anti-WGA antibody. Authentic WGA protein was applied as a positive control (lane 3). **(B)** Immunofluorescence staining. N2a cells infected with AxCAWGA were labeled with anti-WGA antibody followed by Cy3-conjugated anti-rabbit IgG. Intense WGA immunoreactivity was observed in the intracellular granule-like structures.

Adenovirus-mediated WGA expression in the olfactory epithelium *in vivo*

In this study, mice were followed for up to 50 days post-infection (Table 1). A relatively large volume of virus solution (50–70 µl) was required for uniform infection throughout the nasal cavity. There was no evidence of cell loss or tissue damage due to the viral infection. In addition, all of the experimental animals that recovered after the infection remained healthy until being sacrificed, without exhibiting any behavioral abnormalities.

Figure 2A shows transverse cryosections of the olfactory epithelium at 7 days post-infection, immunofluorescently stained with anti-WGA antibody. Abundant WGA expression was detected throughout the olfactory epithelium including nasal septum (S), ventral ectoturbinate (VE), second endoturbinate (II), and third endoturbinate (III). In addition to cell bodies lining along the olfactory epithelium, numerous WGA-containing axonal bundles in the lamina propria were clearly observed.

At higher magnification (Figure 2B), WGA expression was observed in all three types of cells in the olfactory epithelium (olfactory sensory neurons, sustentacular cells and basal cells), as judged from their characteristic shapes and their typical layered positions. Among them, the olfactory sensory neurons in the middle layer of the epithelium exhibited the most predominant WGA immunoreactivity with intracellular granule-like profiles.

Table 1 Time course of WGA expression in olfactory bulb and cortex after nostril infusion of AxCAWGA

Days after infection	*n*	WGA immunoreactivity								
		Olfactory bulb					Olfactory cortex			
		ONL	GL	EPL	MCL	GCL	AON	Tu	Pir	LEnt
7	3	+++	+++	+	++	±	±	–	–	n.d.
12	5	+++	+++	+	++	±	+	±	±	n.d.
20	3	+++	+++	++	+++	+	++	+	±	–
30	4	+++	+++	+	++	+	+	+	+	±
50	3	++	++	+	++	±	±	–	–	–

+++, very high; ++, high; +, weak; ±, faint; –, no expression; n.d., not determined. ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer; AON, anterior olfactory nucleus; Tu, olfactory tubercle; Pir, piriform cortex; LEnt, lateral entorhinal cortex.

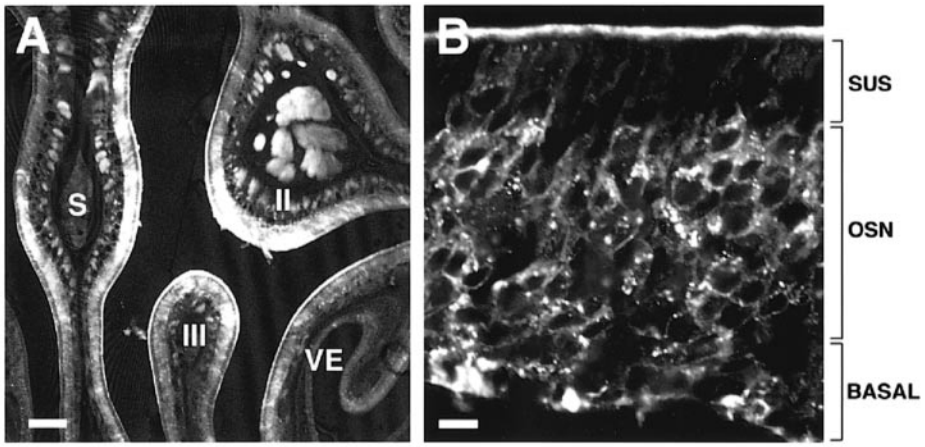

Figure 2 Adenovirus-mediated WGA expression in the olfactory epithelium. **(A)** A coronal section of mouse nose infected with AxCAWGA was stained with anti-WGA antibody. WGA expression was detected throughout the olfactory epithelium. S, septum; VE, ventral ectoturbinate; II, second endoturbinate; III, third endoturbinate. Scale bar, 100 μm. **(B)** In a high-power view, WGA immunoreactivity was observed in all cell types in the olfactory epithelium with predominant expression in the olfactory sensory neurons (OSN). SUS, sustentacular cells; BASAL, basal cells. Scale bar, 5 μm.

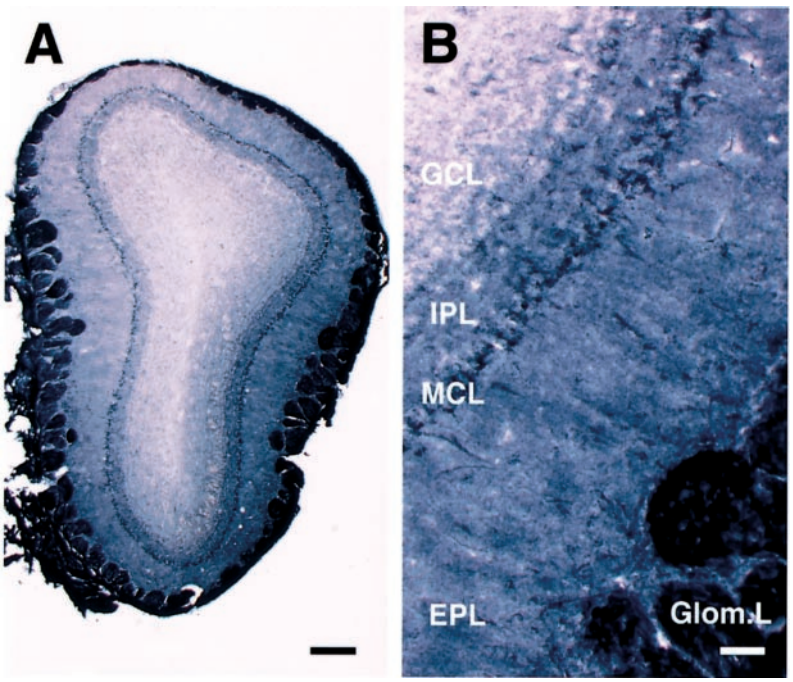

Figure 3 WGA immunoreactivity in the olfactory bulb after 20 days of AxCAWGA infection. **(A)** A coronal section of the olfactory bulb was stained with anti-WGA antibody. A high level of WGA immunoreactivity was detected in axons and nerve terminals of the olfactory sensory neurons and in the mitral cell layer. Scale bar, 200 μm. **(B)** A high-power view shows transsynaptic transfer of WGA from the nerve terminals of the olfactory sensory neurons to the dendrites of mitral/tufted cells in the olfactory glomeruli. Glom. L, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; IPL, internal plexiform layer; GCL, granule cell layer. Scale bar, 40 μm.

Anterograde transsynaptic transfer of WGA to the olfactory centers

Axons leaving from the olfactory epithelium project into spherical neuropil structures, glomeruli, in the main olfactory bulb, where they make synaptic connections with dendrites of the second-order neurons, mitral and tufted cells. In the main olfactory bulb, intense WGA immunoreactivity was detected not only in the olfactory nerve layer and the glomerular layer, but also in the external and internal plexiform layers and the mitral cell layer (Figure 3A). A higher magnification revealed that perikarya of the mitral and tufted cells were strongly WGA-positive (Figure 3B), indicating that WGA protein underwent the anterograde transsynaptic transfer from the sensory axon

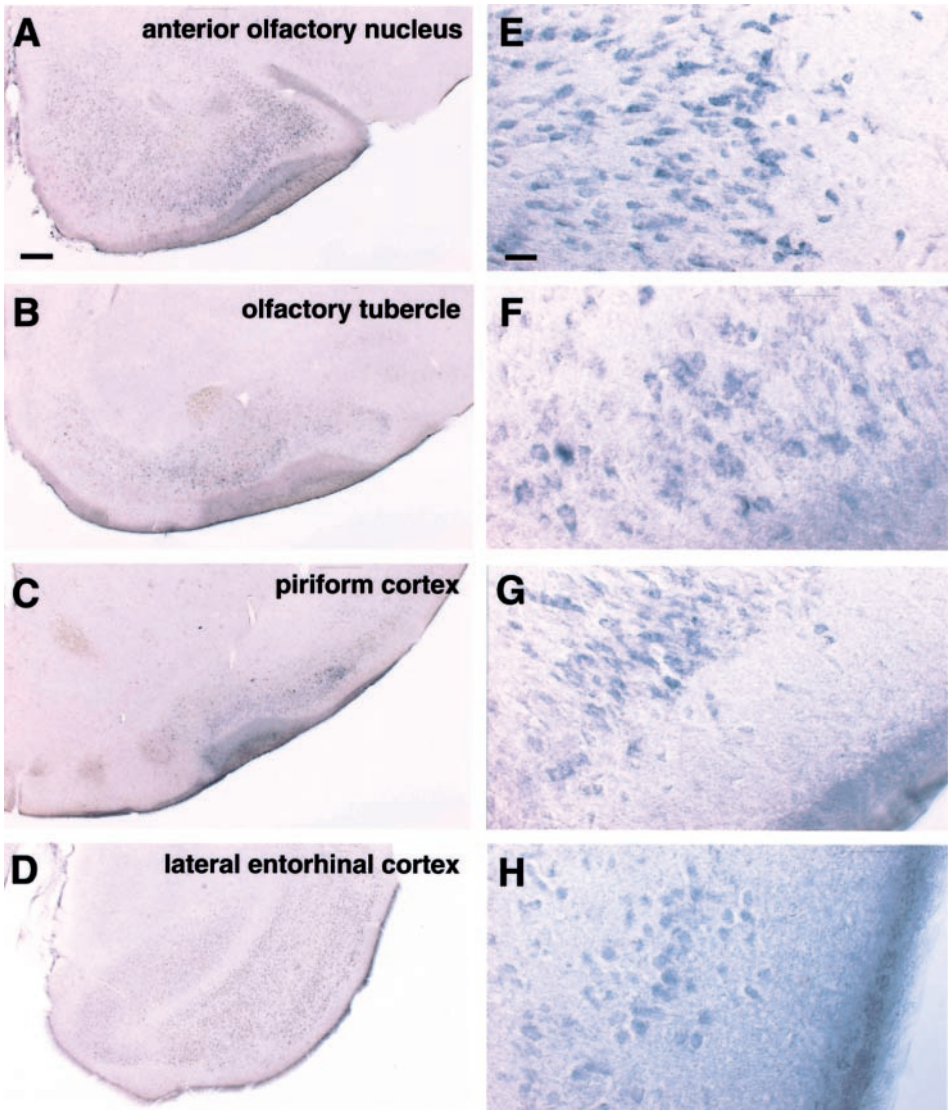

Figure 4 WGA immunoreactivity in the olfactory cortex after 30 days of AxCAWGA infection. **(A–D)** The mitral/tufted cell axons from the main olfactory bulb project to various cortical structures, including the anterior olfactory nucleus **(A)**, the olfactory tubercle **(B)**, the piriform cortex **(C)**, and the lateral entorhinal cortex **(D)**. Scale bar, 200 μm. **(E–H)** High-power views of individual olfactory cortical areas showing the WGA transfer to the third-order neurons. Scale bar, 20 μm.

terminals to the bulbar relay neurons. The presence of WGA protein in the mitral cells was observed at all time points examined, with the strongest signals at 20 days post-infection (Table 1).

The mitral and tufted cells in the main olfactory bulb project their axons to four major terminal fields in the olfactory cortex. The WGA immunoreactivity was observed in all these fields: the anterior olfactory nucleus (Figure 4A,E), the olfactory tubercle (Figure 4B,F), the piriform cortex (Figure 4C,G), and the lateral entorhinal cortex (Figure 4D,H). The most intense signals were detected at 20 and 30 days post-infection (Table 1). Many neurons in these areas of the olfactory cortex contained WGA protein (Figure 4E–H), indicating the transsynaptic labeling of the third-order neurons. These results suggest that WGA protein produced in the olfactory sensory neurons by infection of AxCAWGA was transferred across two synapses from the olfactory epithelium to the bulb, and further to the cortex.

Retrograde labeling of centrifugal modulatory inputs to the olfactory bulb

After 30 days of AxCAWGA infection to the olfactory epithelium, WGA immunoreactivity was detected in neurons of several brainstem nuclei that are known to send a large number of centrifugal modulatory projections to the olfactory bulb. These include cholinergic neurons in the horizontal limb of diagonal band (Figure 5A,D), serotonergic neurons in the midbrain raphe nucleus (Figure

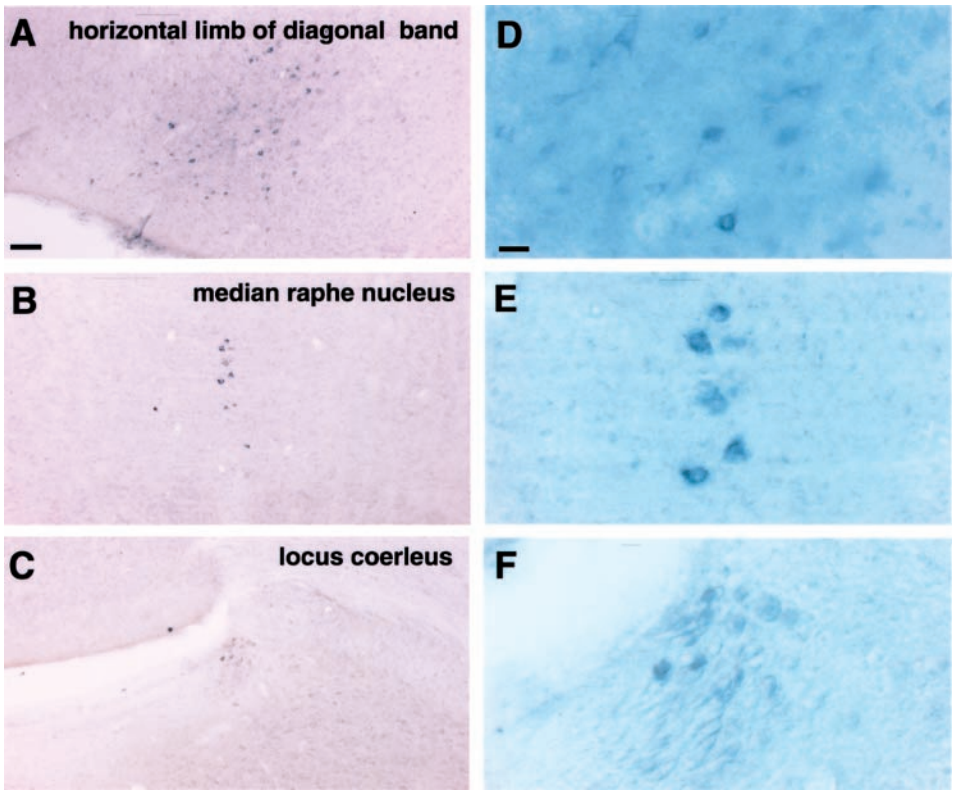

Figure 5 WGA immunoreactivity in the brainstem nuclei after 30 days of AxCAWGA infection. Retrograde transfer of WGA protein from the olfactory bulb to the horizontal limb of diagonal band **(A, D)**, the median raphe nucleus **(B, E)**, and the locus coeruleus **(C, F)**. Neurons in these brainstem regions project massively their axons to the olfactory bulb. Scale bar, 80 μm (A–C); 20 μm (D–F).

5B,E), and noradrenergic neurons in the locus coeruleus (Figure 5C,F). The retrograde labeling was very intense in the horizontal limb of diagonal band and in the raphe nucleus, while only weak immunoreactivity was detected in the locus coeruleus. This result indicates that WGA protein produced in the olfactory epithelium and transported to the main olfactory bulb was taken up into nerve terminals of efferent axons, retrogradely conveyed, and reached to neuronal somata in these distant nuclei.

Discussion

Adenovirus-mediated gene transfer in olfactory sensory neurons

Previously, Zhao *et al.* and Holtmaat *et al.* reported the usefulness of replication-deficient recombinant adenoviral vector system for studies of the rodent olfactory system (Holtmaat *et al.*, 1996; Zhao *et al.*, 1996). In both papers, the adenoviral vector carrying LacZ (β-galactosidase) gene under the control of a strong transcriptional activator (cytomegalovirus promoter/enhancer) was infused into the nasal cavity and efficiently directed transgene expression to the cells in the olfactory epithelium. X-gal staining showed predominant β-galactosidase activity in the olfactory sensory neurons compared with other types of cells such as sustentacular cells and basal cells (Holtmaat *et al.*, 1996; Zhao *et al.*, 1996). A similar preference of the olfactory sensory neurons in adenovirus-mediated transgene expression was observed also in another report by Zhao *et al.* (Zhao *et al.*, 1998) and in this study. This may be due to the difference in effectiveness of adenoviral infection that critically depends on the expression of its cellular receptor CAR (Bergelson *et al.*, 1997; Bergelson, 1999; Asaoka *et al.*, 2000), although the expression pattern of CAR in the rodent olfactory epithelium has not been investigated.

In the two previous papers, the authors used different methods of virus application onto the olfactory epithelium. Zhao *et al.* injected the virus solution (10 μl) with a microsyringe inserted through a small hole in the bone/cartilage at the top of nasal cavity (Zhao *et al.*, 1996). Holtmaat *et al.* performed slow infusion (2.5 μl/min) of virus solution (50 μl) into the nasal cavity using a microinfusion pump (Holtmaat *et al.*, 1996). However, both of these procedures did not result in uniform infection throughout the olfactory epithelium. In contrast, we infected the olfactory sensory neurons by putting a drop of virus solution one by one at the

entrance of the nasal cavity and letting the mice inhale it spontaneously over ~60 min to a total volume of 50–70 μl. With this simple method, we succeeded in relatively uniform infection of the adenoviral vector to the olfactory sensory neurons throughout the epithelium (Figure 2A), which is also evident from thorough labeling of all the glomeruli in the olfactory bulb (Figure 3A).

Olfactory pathways visualized with WGA-expressing adenoviral vector

In this paper, we have demonstrated the feasibility of transsynaptic labeling by using the WGA-expressing adenoviral vector in the mouse olfactory system. By simply infusing the virus solution into mouse nostril, the olfactory neural pathways were clearly visualized with great accuracy and high reproducibility from the olfactory epithelium to the olfactory bulb, and further to the olfactory cortex. In the second-order neurons (mitral cells) in the olfactory bulb, the transgene product, WGA protein, was detected from 7 to 50 days post-infection with a maximal expression at 20–30 days post-infection. In the case of β-galactosidase, it was reported that the transgene expression in the olfactory sensory neurons persisted for 8–12 days and decreased at 25 days (Holtmaat *et al.*, 1996). These results indicate that the WGA protein was so stable to be sufficiently accumulated in relay neurons, resulting in clear visualization of the olfactory neural pathways.

Granule cells in the olfactory bulb are GABAergic interneurons that play important roles in lateral inhibition between neighboring mitral cells. The granule cells and the mitral cells make dendrodendritic reciprocal synapses. Transsynaptic labeling of the granule cells in the adenovirus-mediated WGA-expressing mice in this paper (Figure 3A) was significantly weaker than that in the OMP-WGA transgenic mice in our previous study (Yoshihara *et al.*, 1999). One possible explanation is that WGA transfer across the dendrodendritic synapse may be not so efficient that chronic, long-lasting expression would be required for definite labeling of the granule cells.

The mammalian olfactory bulb receives profuse innervation of centrifugal fibers from the nonolfactory subcortical modulatory systems. In addition to the anterograde transfer of WGA from the olfactory epithelium to the bulb and further to the cortex, we observed the retrograde transsynaptic labeling from the olfactory bulb to these brainstem nuclei (Figure 6). WGA immunoreactivity was very intense in cholinergic neurons in the horizontal limb of diagonal band and serotonergic neurons in the midbrain raphe nucleus, but faint in noradrenergic neurons in the locus coeruleus. This difference in WGA appearance among three types of neurons may be attributable to different termination fields in the olfactory bulb. The cholinergic and serotonergic terminals are distributed densely in the glomerular layer (McLean and Shipley, 1987; Shipley *et al.*, 1995) where a large amount of WGA is transported directly from the olfactory epithelium. In contrast, the noradrenergic terminals are found mostly in the granule cell layers (Halasz and Shepherd, 1983; McLean *et al.*, 1989) where WGA is scarcely present.

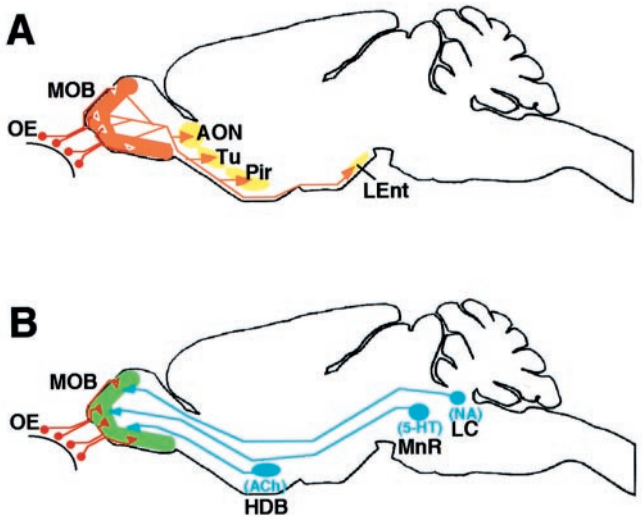

Figure 6 Schematic diagrams of mouse olfactory pathways visualized with WGA-expressing adenoviral vector. **(A)** Anterograde labeling. WGA protein was produced in the olfactory sensory neurons (red) in the olfactory epithelium (OE), transferred to the second-order neurons (orange) in the main olfactory bulb (MOB), and further transported to the olfactory cortical areas (yellow) including the anterior olfactory nucleus (AON), the olfactory tubercle (Tu), the piriform cortex (Pir), and the lateral entorhinal cortex (LEnt). **(B)** Retrograde labeling. WGA protein in the main olfactory bulb (green) was taken up by the terminals of centrifugal axons and retrogradely transported to three brainstem nuclei (blue): the horizontal limb of diagonal band (HDB), the median raphe nucleus (MnR), and the locus coeruleus (LC). ACh, acetylcholine; 5-HT, serotonin; NA, noradrenaline.

Compared to the conventional method in which WGA protein was infused into the nostril (Shipley, 1985; Itaya, 1987), we could detect more strongly and reliably the transsynaptically transferred WGA protein in the olfactory cortical neurons and the median raphe nucleus. This may be due to the efficient infection of adenovirus to the olfactory sensory neurons and to the extremely strong promoter elements (CAG promoter) we used for robust expression of WGA.

Further applications of the WGA-expressing adenoviral vector system

Detailed knowledge of the neural connectivity patterns is indispensable for understanding a wide range of brain functions, especially higher cognitive functions that involve combinations of intricate neural networks widely distributed in the brain. The technique described in this paper enabled us to deliver plant lectin WGA to olfactory sensory neurons with the aid of the adenoviral vector system and to visualize the olfactory neural pathways. This method will be useful not only in the olfactory system, but also in various neural systems focusing on the development, anatomy and

functions of the brain. Because of the wide host range of adenovirus, furthermore, this technique will be most useful for the application to other mammalian species such as monkeys, cats and ferrets that are not amenable to the transgenic technology, but are useful in neuroscience research (Bohn *et al*., 1999; Lakatos *et al*., 2000; Lawrence *et al*., 1999; Liu *et al*., 2000).

In this paper, we utilized a CAG promoter for the expression of WGA. Under the control of the CAG promoter, a robust expression of the downstream transgene is driven in any type of mammalian cells, including neurons (Miyake *et al*., 1996). In contrast, cell type-specific expression of the WGA transgene will be induced by choosing appropriate promoter elements. In a preliminary study, we have succeeded in adenovirus-mediated WGA expression in a subpopulation of the olfactory sensory neurons by using a promoter element of olfactory zone-specific cell adhesion molecule, OCAM (Yoshihara *et al*., 1997; N.K., T.M. and Y.Y., unpublished result). Thus, 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 ways.

A further refinement of the technique would be to introduce a bicistronic transgene consisting of WGA and another reporter molecule with internal ribosome entry sequence (IRES) in-between. For example, the WGA-IRES-GFP transgene could be used conveniently for discrimination of the first-order neurons (WGA- and GFP-producing neurons) from the second- and third-order neurons (transferred WGA-containing neurons). Furthermore, if a retrograde direction-specific transneuronal tracer such as tetanus toxin C fragment (TTC) can be used as a transgene (Coen *et al*., 1997), a simultaneous expression of three molecules (WGA, TTC and GFP) will provide us with more detailed information on neuronal connectivity patterns originating from the infected neurons. In conclusion, the use of a WGA-expressing adenoviral vector system should greatly facilitate studies on the anatomical and functional organization of the developing and mature nervous system.

Acknowledgements

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