Adenoviral Vector-Mediated Expression of B-50/GAP-43 Induces Alterations in the Membrane Organization of Olfactory Axon Terminals *In Vivo*

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B-50/GAP-43 is an intraneuronal membrane-associated growth cone protein with an important role in axonal growth and regeneration. By using adenoviral vector-directed expression of B-50/GAP-43 we studied the morphogenic action of B-50/GAP-43 in mature primary olfactory neurons that have established functional synaptic connections. B-50/GAP-43 induced gradual alterations in the morphology of olfactory synapses. In the first days after overexpression, small protrusions originating from the preterminal axon shaft and from the actual synaptic bouton were formed. With time the progressive formation of multiple ultraterminal branches resulted in axonal labyrinths

composed of tightly packed sheaths of neuronal membrane. Thus, B-50/GAP-43 is a protein that can promote neuronal membrane expansion at synaptic boutons. This function of B-50/GAP-43 suggests that this protein may subserve an important role in ongoing structural synaptic plasticity in adult neurons and in neuronal membrane repair after injury to synaptic fields.

Key words: growth-associated protein B-50/GAP-43; adenoviral vector-mediated gene transfer; olfactory system; transgenic mice; axon morphology; synaptic plasticity

Growing evidence suggests that the neuronal growth-associated protein B-50/GAP-43 (also known as F1, pp46, and neuromodulin) is an important regulator of structural axonal plasticity. Continued expression of B-50/GAP-43 in the nervous system of transgenic mice results in ectopic hippocampal and motoneuron projections (Aigner et al., 1995) and in the formation of abnormally shaped olfactory axon endings (Holtmaat et al., 1995). During neuroembryogenesis, B-50/GAP-43 first appears in differentiating neuronal precursors that have just begun to elaborate nerve fibers (Biffo et al., 1990; Dani et al., 1991). The levels of B-50/GAP-43 decrease in most neurons during postnatal development, although significant expression is observed in parts of the nervous system that maintain a high level of synaptic plasticity during adulthood (Benowitz et al., 1988; Neve et al., 1988). In the hippocampus of adult rats, kainic acid-induced mossy fiber sprouting is preceded by upregulation of B-50/GAP-43 (Cantallors and Routtenberg, 1996). Lesion-induced upregulation of B-50/GAP-43 in the peripheral nervous system (PNS) is accompanied by axon regeneration (Benowitz et al., 1981; Skene and

Willard, 1981a,b; Benowitz and Lewis, 1983; Bisby, 1988; Verhaagen et al., 1988; Tetzlaff et al., 1989). Upregulation of B-50/GAP-43 in the injured CNS is not accompanied by regeneration (Doster et al., 1991; Tetzlaff et al., 1991) unless a peripheral nerve graft is present (Vaudano et al., 1995; Chong et al., 1996). One explanation for these findings is that increased expression of B-50/GAP-43 is not sufficient to stimulate regenerative sprouting into the inhibitory environment of the CNS but that the PNS graft provides a substrate permissive for regrowth of B-50/GAP-43-expressing neurites.

In the transgenic mice, B-50/GAP-43 was overexpressed in neurons by using constructs that contained the promoters for *Thy-1.2* (Aigner et al., 1995) or olfactory marker protein (*OMP*) (Holtmaat et al., 1995). Both promoters gain full transcriptional activity during the first 2 weeks after birth. During this period, endogenous B-50/GAP-43 expression is still quite high but is declining in most neurons. This approach revealed that persistent expression of B-50/GAP-43 throughout postnatal development can result in extended terminal fields of PNS and CNS neurons in adulthood (Aigner et al., 1995). In transgenic mice it is difficult to define the role of B-50/GAP-43 in structural plasticity of mature neurons, because the promoters used in these mice direct expression of B-50/GAP-43 in neurons throughout their postnatal development.

In this study, adenoviral vectors were used to target B-50/GAP-43 expression to the olfactory neuroepithelium of adult mice to study its role in the structural plasticity of mature neurons that have established functional synaptic connections. Adenovirus is a suitable vector to deliver a foreign gene to mature OMP-expressing olfactory neurons of adult mice *in vivo* (Holt-

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maat et al., 1996; Zhao et al., 1996). These neurons reside in the olfactory neuroepithelium in the nasal cavity and connect to the olfactory bulb where they terminate in structures termed glomeruli. Viral vector-mediated gene transfer was combined with confocal laser scanning microscopy and ultrastructural and immunoelectron microscopical analysis to examine the effect of B-50/GAP-43 on neuronal morphology at different intervals after B-50/GAP-43 overexpression. We demonstrate that B-50/GAP-43 induces ultraterminal branches at or just proximal to primary olfactory synapses. Interestingly, B-50/GAP-43-induced growth progresses into the formation of complex structures, reminiscent of axonal labyrinths that have been observed previously in central projections of peripherally axotomized sensory nerve cells (Knyihar and Csillik, 1976). The ultrastructural findings indicate that an important property of B-50/GAP-43 is to promote the expansion of axolemma at nerve endings and provide evidence that this protein may be a critical determinant of ongoing structural plasticity in synaptic boutons during adulthood.

MATERIALS AND METHODS

Generation of adenoviral vectors for LacZ and B-50/GAP-43. A plasmid was constructed containing the human adenovirus type 5 (Ad-5) inverted terminal repeat (adenovirus map units 0–1.25) and a region of the Ad-5 genome ranging from 9.2 to 15.5 map units (pAd309dE1.sl). The human cytomegalovirus immediate early (CMV) promoter-LacZ reporter gene linked to a SV40 poly(A) sequence was cloned between map units 1.25 and 9.2, resulting in plasmid pAdCMVLacZ. This plasmid was used to generate pAdCMVB-50, carrying the coding region of the B-50/GAP-43 gene (see Fig. 1*A,B*). To this end the coding sequence of B-50/GAP-43 was inserted into the multiple cloning site of pcDNA5.0 (Invitrogen, San Diego, CA), yielding pcDNAB-50. The LacZ-SV40 poly(A) fragment in pAdCMVLacZ was replaced by the B-50-SV40 poly(A) fragment, derived from pcDNAB-50. The generation of the recombinant adenoviral vectors Ad-LacZ and Ad-B-50/GAP-43 was performed as described previously using the adenoviral vector producer 911 cells (see Fig. 1) (Giger et al., 1997; Fallaux et al., 1996; Hermens et al., 1997). The titers of the recombinant viral vector stock solutions were determined by plaque assay (Graham and Prevec, 1991) and are expressed as plaque-forming units (pfu).

Expression of B-50/GAP-43 in *vero* cells and Western blot analysis. Vero (African green monkey kidney) cells were plated in 10 cm dishes and allowed to grow to ~70% confluence in DMEM containing 10% inactivated fetal calf serum (FCS) and 1 gm/l glucose at 37°C. The culture medium was removed, and 5 ml of inoculation medium containing 5×10^7 , 10^8 or 5×10^8 pfu Ad-B-50/GAP-43 or 5×10^8 pfu Ad-LacZ in 2% FCS was added. After 1.5 hr, the inoculation medium was replaced by culture medium containing 10% FCS and kept in culture for 48 hr. The cells were washed two times with PBS and removed from the dish using a denaturing electrophoresis sample buffer (Zwiers et al., 1976). The mouse olfactory bulbs were dissected to serve as a reference sample for the immunodetection of native B-50/GAP-43. The bulbs were homogenized in TBS (10 mM Tris-HCl, pH 7.5, 0.9% NaCl) at 4°C, and the homogenate was stored at –20°C in denaturing sample buffer. Before electrophoresis, proteins in sample buffer were heated for 10 min at 80°C. Proteins were separated by electrophoresis in an 11% polyacrylamide-SDS gel and transferred to nitrocellulose membranes. The membrane was preincubated in TBS with 5% FCS at room temperature and immunostained with polyclonal antibodies against B-50/GAP-43 under standard conditions (Oestreicher et al., 1983).

Animals and surgery. Mice (FVB/N) were 5–8 month of age, weighed ~30 gm, and were maintained on a 12 hr light/dark cycle in the breeding facility of the Academic Medical Center in Amsterdam. Intranasal infusion of Ad-B-50/GAP-43 ($n = 25$) and Ad-LacZ ($n = 8$) was performed according to a previously published procedure (Holtmaat et al., 1996). In short, in anesthetized mice (Hypnorm, Janssen Pharmaceutical Ltd, Oxford, England; together with Dormicum, Roche Nederland B.V., Mijdrecht, The Netherlands) a polyethylene tube was inserted into the right nostril to a depth of 7 mm until it just reached the olfactory neuroepithelium, and 20 μ l of virus buffer, containing 5×10^8 pfu of Ad-LacZ or Ad-B-50/GAP-43, was infused over a period of 20 min.

Nine days after viral vector infusion, mice ($n = 11$) were injected

intravenously with 20 μ g/gm body weight of bromodeoxyuridine (BrdU) (Boehringer Mannheim, Mannheim, Germany) dissolved in sterile saline. At 3 d after BrdU injection, the mice were perfused, and tissue was processed as described previously (Holtmaat et al., 1995). The number of BrdU-labeled nuclei per millimeters of olfactory epithelium was determined with a computerized image analysis system. Two 2–3 mm stretches of septal epithelium at the site of adenovector injection per animal, ~100 μ m apart, were analyzed.

The production and characterization of the B-50/GAP-43 transgenic mice has been described previously (Holtmaat et al., 1995). In these transgenic mice the regulatory elements of the OMP gene were combined with the coding sequence of B-50/GAP-43 in a transgene that directs expression of B-50/GAP-43 specifically to mature olfactory neurons.

Immunohistochemistry and confocal microscopy. Adenoviral vector-infused mice (at 3, 5, 8, or 12 d after viral vector administration) and transgenic mice were anesthetized and perfused with 50 ml PBS, pH 7.4, followed by 100 ml of 4% paraformaldehyde (PFA). The bulbs were dissected and post-fixed for 12 hr at 4°C. Pairs of vibratome sections of olfactory bulbs were prepared. One section of each pair was stored in PBS and processed further for immunocytochemistry, and the adjacent section was post-fixed in 5% glutaraldehyde for epon embedding [or in 1% PFA to allow preembedding detection of β -galactosidase (β -gal)].

The sections stored in PBS were rinsed in TBS/TX-100 (TBS containing 0.5% Triton X-100) and preincubated with TBS/gelatin/TX-100 (TBS/TX-100 containing 0.25% gelatin) for 30 min. B-50/GAP-43 was detected with affinity-purified polyclonal rabbit antibodies derived from antiserum no. 8921 (dilution 1:1000) (prepared according to Oestreicher et al., 1983), OMP with polyclonal goat antibodies (antiserum no. 255; dilution 1:5000) (Keller and Margolis, 1975), and β -gal with monoclonal mouse antibodies (Gal 13, dilution 1:2000; Sigma, St. Louis, MO). Binding of primary antibodies was visualized with dichlorotriazinylamino fluorescein-conjugated anti-rabbit or CY3-conjugated anti-goat or anti-mouse IgGs (Jackson ImmunoResearch Laboratories, West Grove). Sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined on a Zeiss confocal laser scanning microscope equipped with lasers and filters allowing emission at 488 and 543 nm. Appropriate filters were used to prevent cross-talk. A stack of eight focal planes (1 μ m intervals) was imaged for all fluorophores, using oil immersion objectives, after which one single projection was generated.

Tissue preparation for ultrastructural analysis. Because Ad-B-50/GAP-43 and Ad-LacZ transduce a proportion of primary olfactory neurons and in transgenic mice a subpopulation of primary olfactory neurons expresses the transgene, not all glomeruli contain B-50/GAP-43- or β -gal-positive fibers. Therefore, camera lucida drawings of the immunostained sections containing B-50/GAP-43- or β -gal-positive axon profiles were used to select areas from the adjacent sections (stored in 5% glutaraldehyde or 1% PFA) to be processed for electron microscopy. For preembedding β -gal staining, sections were freeze-thaw pretreated as described previously (Boersma et al., 1993) to improve penetration of antibodies. The immunostaining was performed as described above, but TX-100 was omitted in all solutions and biotinylated horse anti-mouse IgGs were used as secondary antibodies (Vector). Visualization of the antibodies was performed with the avidin-biotin-peroxidase method (Vectastain ABC kit, Vector), using diaminobenzidine (DAB) (0.5 mg/ml 10 mM Tris, pH 7.6) as the chromogen. To reduce ultrastructural damage attributable to the DAB reaction, the sections were post-fixed in 2.5% glutaraldehyde and 1% PFA before the DAB incubation, the concentration of H₂O₂ was lowered to 0.005%, and the incubation time was reduced to 7 min. The 5% glutaraldehyde-fixed sections and β -gal-immunostained sections were rinsed and immersed in 1% osmium tetroxide (Merck) with 1.5% potassium hexacyanoferrate(III) in PBS for 20 min at 4°C. The tissue was contrasted with 0.5% uranyl acetate in 60% ethanol for 25 min, further dehydrated, and gradually immersed in pure propylene oxide and embedded in Epon.

For postembedding immunogold labeling, 2% glutaraldehyde with 4% PFA was used as fixative. The bulbs were dissected and post-fixed for 12 hr at 4°C. Vibratome sections (100 μ m) of the olfactory bulbs were cut, and small blocks of tissue (0.3 $\times$ 2 mm) containing the glomerular area were dissected. Freeze substitution and Lowicryl embedding were performed as described previously (Van Lookeren Campagne et al., 1991). In short, the tissue blocks were immersed in 10, 20, and 30% glycerol in PBS for 30 min, subsequently frozen ultra-rapidly in liquid propane (–196°C), and stored in liquid nitrogen until further use. The tissue was transferred to methanol containing 0.5% uranyl acetate at –90°C in a

freeze substitution apparatus (Reichert-Jung, Wien, Austria) for 25 hr. Subsequently the temperature was raised stepwise to -50°C (5°C/hr), and the methanol was replaced gradually by Lowicryl HM20 (Chemische Werke Lowi, Waldkraiburg, Germany). Pure Lowicryl was allowed to infiltrate for 40 hr and was polymerized under ultraviolet light for 70 hr. Ultrathin sections (60 nm) were cut on a Reichert-Jung Ultra Cut using a diamond knife (Diatome) and collected on formvar and carbon-coated nickel grids. Immunogold labeling of Lowicryl sections was performed according to the protocol provided by Aurion (Wageningen, The Netherlands). Before the incubation the sections were rinsed in TBS containing 50 mM glycine. Antibodies were diluted in TBS/0.5% bovine serum albumin/0.2% fish gelatin to reduce background staining. Serial sections were incubated overnight at 4°C with anti-OMP (no. 255; 1:1000) or anti-B-50/GAP-43 (no. 8921; 1:500). The goat anti-rabbit and rabbit anti-goat IgGs were coupled to ultra-small gold particles (Aurion). The gold was further silver-enhanced (silver enhancement kit, Aurion). Electron microscopy was performed on a Philips CM-10.

RESULTS

An adenoviral vector encoding B-50/GAP-43 directs expression of biologically active B-50/GAP-43

Replication-deficient adenoviral vectors for the *Escherichia coli* *LacZ* gene (designated Ad-*LacZ*) and for B-50/GAP-43 (designated Ad-B-50/GAP-43) were constructed according to a previously published protocol (Giger et al., 1997; Hermens et al., 1997). The procedure to generate the viral vector for B-50/GAP-43 is depicted schematically in Figure 1*A,B*.

First, it was necessary to determine whether Ad-B-50/GAP-43 can be used to direct the expression of intact and biologically active B-50/GAP-43. Earlier studies (Zuber et al., 1989; Widmer and Caroni, 1993; Verhaagen et al., 1994) have revealed that expression of B-50/GAP-43 in non-neuronal cells induces filopodia and cellular processes. We used this bioassay and Western blot analysis to examine the functional expression of intact B-50/GAP-43 via Ad-B-50/GAP-43. African green monkey kidney cells (vero cells) were infected with Ad-B-50/GAP-43 or Ad-*LacZ* at a multiplicity of infection of 10, 50, and 100. Two days later the cultured cells either were harvested to perform Western blot analysis or fixed and immunohistochemically stained for β -gal or B-50/GAP-43 to examine changes in cell shape. The Western blot (Fig. 1*C*) revealed that Ad-B-50/GAP-43 directs the expression of intact B-50/GAP-43 with the same apparent molecular weight as B-50/GAP-43 from mouse olfactory bulb. Light microscopical analysis of Ad-B-50/GAP-43-transduced vero cells revealed the typical and previously reported B-50/GAP-43-induced changes in cell shape, including ruffled membranes, filopodia, and the formation of cellular processes, extending from the cell over a distance of several cell diameters (data not shown). In Ad-*LacZ*-infected cells, such morphological changes were never observed. Thus, B-50/GAP-43 expressed via Ad-B-50/GAP-43 induced the expected morphological alterations in non-neuronal cells.

Expression of B-50/GAP-43 via an adenoviral vector in the olfactory neuroepithelium of adult mice

To determine whether Ad-B-50/GAP-43 can serve as a vector to express B-50/GAP-43 in primary olfactory neurons *in vivo* and to establish that after viral vector-mediated gene transfer to the olfactory epithelium the neuronal turnover process that normally occurs in this neuroepithelium remained unchanged, three groups of mice were injected with either virus buffer or virus buffer containing 5×10^8 pfu Ad-*LacZ* or 5×10^8 pfu Ad-B-50/GAP-43 (virus buffer, $n = 7$; Ad-*LacZ* and Ad-B-50/GAP-43, $n = 4$). The viral vectors were applied unilaterally by gradual instillation, using a microinfusion pump (Holtmaat et al., 1996). At 9 d after viral vector application, all mice were injected with BrdU to

Figure 1. Construction of Ad-B-50/GAP-43 and Western blot analysis of vero cells expressing B-50/GAP-43 via Ad-B-50/GAP-43. *a*, Structure of pAdCMVB-50. The plasmid contains an expression cassette consisting of the human cytomegalovirus immediate early (CMV) promoter linked to the B-50/GAP-43 open reading frame (ORF) and the SV40 polyadenylation sequence [SV40 poly(A)] of the simian virus 40 early gene. The expression cassette was cloned between the inverted terminal repeat (ITR) (map units 0–1.25; the viral genome encapsidation signal) and the 9.2–15.5 map units (m.u.) sequence of the adenovirus type 5 genome. Furthermore, the plasmid contains the ampicillin resistance gene (*Amp^R*) and an origin of replication (*Ori*). *b*, To generate the viral vector Ad-B-50/GAP-43, pAdCMVB-50 was linearized with *SalI* and transfected into producer 911 cells together with the *ClaI* and *XbaI* truncated Ad5dl309 genomic DNA. *c*, Western blot analysis detecting B-50/GAP-43 expression in Ad-*LacZ*-infected vero cells [5×10^8 pfu (lane 1)], olfactory bulb (lane 2), and Ad-B-50/GAP-43-infected vero cells [5×10^7 pfu (lane 3), 10^8 pfu (lane 4), 5×10^8 pfu (lane 5)]. Vero cells were infected with the viral vectors for 1.5 hr, and after 48 hr the cells were harvested. Proteins extracted from these vero cells were separated by SDS-PAGE, blotted on nitrocellulose, and probed with anti-B-50/GAP-43 antibodies. A similarly treated sample of mouse olfactory bulb proteins was run (lane 2) as a reference sample. Note that Ad-*LacZ*-infected vero cells do not express B-50/GAP-43 and that B-50/GAP-43 from mouse olfactory bulb and B-50/GAP-43 expressed in vero cells via Ad-B-50/GAP-43 migrate at the same position in the gel.

Figure 2. Ad-B-50/GAP-43- and Ad-LacZ-directed expression of B-50/GAP-43 and β -gal in mature olfactory neurons. *A, B*, Low power photomicrographs of transversal sections of mouse olfactory epithelia stained for β -gal (*A*) and B-50/GAP-43 (*B*) showing the patchy expression pattern of these molecules throughout the neuroepithelium 3 d after infusion of Ad-LacZ (*A*) and 12 d after infusion of Ad-B-50/GAP-43 (*B*). Note that areas containing high numbers of transduced cells (arrowheads in *B*) are alternated by areas without transduced cells (arrows in *B*). *C–E*, Confocal laser scanning micrographs of the expression of B-50/GAP-43 (*C, D*) and OMP (*E*) in olfactory neuroepithelia of Ad-LacZ (*C*) and Ad-B-50/GAP-43-injected mice (*D, E*) at 12 d after viral vector infusion. In mice infused with Ad-LacZ, B-50/GAP-43 expression is restricted to the normal population of immature cells in the basal portion of the epithelium (arrowhead in *C*). In contrast, in Ad-B-50/GAP-43-infused mice B-50/GAP-43-positive cells are detected in a scattered pattern through the olfactory neuroepithelium. Double-labeling for B-50/GAP-43 and OMP reveals numerous mature OMP-positive neurons coexpressing B-50/GAP-43 (arrows in *D, E*), indicating that mature neurons are efficiently transduced by Ad-B-50/GAP-43. Scale bar (shown in *E*): *A*, 500 μ m; *B*, 125 μ m; *C–E*, 50 μ m.

monitor the mitotic activity in the olfactory epithelium. The 9 d time point was chosen because lesion-induced cell death in the olfactory epithelium results in a clearly enhanced labeling index in the dividing cells in the basal region of the epithelium as part of a regenerative response in the second postlesion week (Schwob et al., 1992). Mice were killed 3 d later after BrdU administration, that is, at 12 d after viral vector infusion. As reported previously, sections through the turbinates of mice infected with Ad-LacZ contained individual cells and small groups of mature olfactory neurons and sustentacular cells scattered throughout the olfactory epithelium expressing β -gal (Fig. 2*A*) (Holtmaat et al., 1996). Immunohistochemical detection of the distribution of B-50/GAP-43 in the Ad-B-50/GAP-43-infected group revealed a transduction pattern that was comparable to the pattern of β -gal staining in the Ad-LacZ-infected group (Fig. 2*B*). The Ad-B-50/GAP-43-infected areas contained many B-50/GAP-43-expressing mature, OMP-positive, olfactory neurons (Fig. 2*D,E*). In contrast, mice that received virus buffer alone or Ad-LacZ exhibited the normal pattern of B-50/GAP-43 expression restricted to immature olfactory neurons located in the basal region of the epithelium (Fig. 2*A*) (Verhaagen et al., 1989). Quantitative analysis of the number of mitotic divisions in the olfactory epithelium, as based on the number of cells that incorporated BrdU, showed

equivalent numbers of BrdU-positive cells in the virus buffer-injected group and in the group treated with adenoviral vectors (virus buffer, 19 ± 13 cells/mm; Ad-LacZ, 34 ± 15 cells/mm; Ad-B-50/GAP-43, 30 ± 15 cells/mm; *t* test revealed no significant difference between virus buffer and Ad-LacZ, $p = 0.11$, or Ad-B-50/GAP-43, $p = 0.22$), suggesting that adenoviral vector treatment does not significantly affect the rate of turnover. Taken together, these observations show that adenoviral vectors can be used to target B-50/GAP-43 to mature, OMP-positive, primary olfactory neurons and that adenoviral vector-mediated gene transfer does not result in the induction of endogenous B-50/GAP-43 gene expression in mature olfactory neurons or in detectable changes in the neuronal turnover process that occurs in the olfactory neuroepithelium throughout adulthood.

B-50/GAP-43 and plasticity of primary olfactory projections

The olfactory receptor neurons form the primary olfactory pathway, projecting from the olfactory neuroepithelium to the olfactory bulb, where their axons terminate in structures termed glomeruli. The glomeruli primarily contain neuropil consisting of the axons and synapses of olfactory receptor neurons on the projections of second-order neurons, the juxtaglomerular and mitral

Figure 3. B-50/GAP-43-induced morphogenic changes occur in mature OMP-positive olfactory neurons. Confocal laser scanning micrographs were taken from olfactory bulbs at 12 d after infusion of Ad-LacZ (*A*) or Ad-B-50/GAP-43 (*B–D*) and immunohistochemically stained for β -gal (*A*) or B-50/GAP-43 (*B*) or double-immunolabeled for OMP (*C*) and B-50/GAP-43 (*D*). Thin, long β -gal-expressing axons are present in a scattered pattern throughout the glomerulus and are often topped with small synaptic boutons (*A*, arrows). In contrast, B-50/GAP-43-expressing fibers exhibit large, morphologically altered axon endings that are formed predominantly at the edge of the glomerulus (*B*, arrows). Aberrant axon endings (arrowheads) are double-stained for B-50/GAP-43 (*D*) and OMP (*C*), indicating that these axon profiles represent morphologically changed mature olfactory nerve endings. Scale bar (shown in *D*): *A*, *B*, 25 μ m; *C*, *D*, 30 μ m.

cells. Adenoviral vector-mediated gene transfer to primary olfactory neurons was combined with confocal laser scanning microscopy and ultrastructural analysis to investigate the role of B-50/GAP-43 in the control of presynaptic plasticity of olfactory neurons. The morphology of individual axons expressing either β -gal or B-50/GAP-43 was studied by confocal laser scanning microscopy at 3, 5, 8, and 12 d after adenoviral vector administration (Figs. 3, 4). At 3 and 5 d, a scattered pattern of labeled olfactory nerve fibers and small synaptic boutons were visible throughout the neuropil of individual glomeruli in both Ad-LacZ- and Ad-B-50/GAP-43-infected mice (Fig. 4). After 3 d, with the spatial resolution of the confocal laser scanning microscope, the B-50/GAP-43-expressing fibers exhibited an appearance similar to control axons expressing β -gal. Between 5 and 12 d, however, changes in the distribution of the B-50/GAP-43-expressing fibers in the glomerular neuropil and in the morphological appearance of olfactory nerve endings became apparent (Figs. 3, 4). During this period, many of the B-50/GAP-43-positive axon endings became located at the glomerular edge. Axons with unusual, large grape-like nerve endings were predominantly observed at the rim of individual glomeruli (Figs. 3*B*, 4*E*). The β -gal-expressing axons displayed a normal axonal morphology, similar to the structure of olfactory axons previously revealed by a classic Golgi staining (Fig. 3*A*) (Halász and Greer, 1993; Holtmaat et al., 1995). Double-labeling of the B-50/GAP-43-positive morphologically changed olfactory axon endings demonstrated that these axons express OMP, a marker protein of mature olfactory neurons (Fig.

3*C,D*). This is consistent with the observations in the olfactory neuroepithelium showing that the Ad-B-50/GAP-43-transduced olfactory neurons have a mature, OMP-positive phenotype (Fig. 2*B,C*). Likewise, large puncta of OMP in the glomeruli always colocalized with B-50/GAP-43, indicating that the morphological changes occur exclusively in B-50/GAP-43-expressing axons. These observations show that reexpression of B-50/GAP-43 via an adenoviral vector in mature olfactory neurons induces growth of their terminal axon segments. Subsequently, morphologically altered axon endings are found at the rim of the olfactory neuropil that only occasionally penetrate for a very short distance between the juxtglomerular cells (Figs. 3*B*, 4*E*).

B-50/GAP-43-induced ultrastructural changes in primary olfactory synaptic boutons

The abnormally shaped terminal axon profiles, as identified by confocal microscopy at 8 and 12 d after adenoviral vector application, display a striking resemblance to the structural changes observed in transgenic mice, with continued overexpression of B-50/GAP-43 in mature olfactory neurons directed by the *OMP* promoter. To study the ultrastructure of olfactory axons expressing B-50/GAP-43, electron microscopy was performed on olfactory bulb glomeruli of transgenic mice derived from two independent transgenic mouse lines, L29 ($n = 4$) and L30 ($n = 4$) (L29 displays overexpression of B-50/GAP-43 in a relatively large number of primary olfactory neurons; L30 contains a relatively small set of B-50/GAP-43-positive primary olfactory neurons),

Figure 4. B-50/GAP-43-overexpressing primary olfactory axon endings grow toward the edge of glomeruli in the olfactory bulb. Confocal laser scanning micrographs were taken from mice infused with virus buffer (*A*) or with Ad-B-50/GAP-43 (*B–E*). Sections were stained with anti-B-50/GAP-43 antibodies at 3 (*B*), 5 (*C*), 8 (*D*), and 12 (*A*, *E*) d after infusion of Ad-B-50/GAP-43. B-50/GAP-43 is virtually absent in glomeruli in olfactory bulbs of control mice infused with virus buffer alone (*A*). At 3 and 5 d after infusion of Ad-B-50/GAP-43, B-50/GAP-43 is present in primary olfactory axons scattered throughout the neuropil of individual glomeruli (*B*, *C*), whereas at 8 and 12 d after infusion B-50/GAP-43-positive fiber endings become more and more located at the rim of individual glomeruli (*D*, *E*). This indicates that primary olfactory axon endings translocate from the glomerular neuropil to the edge of the glomerulus, where they form enlarged axonal terminals (arrows in *D*, *E*). Scale bar, 50 μ m.

and on glomeruli of mice at 3 ($n = 4$), 5 ($n = 4$), and 12 ($n = 4$) d after intranasal administration of Ad-B-50/GAP-43. Nontransgenic littermates ($n = 4$) and Ad-LacZ-infected mice ($n = 4$) were included as controls.

Immunoelectron microscopy on Lowicryl-embedded tissue of transgenic mice revealed numerous olfactory axon profiles terminating in highly B-50/GAP-43-immunostained structures containing large amounts of axolemma organized in concentric sheaths (Fig. 5). The concentrically organized axon profiles were always intensely labeled for B-50/GAP-43, indicating that the appearance of these morphological alterations is closely associated with the expression of B-50/GAP-43. Observations in adjacent sections demonstrated that these structures are derived from mature primary olfactory axons because they also express OMP (Fig. 5*D,E*). In the transgenic mice, conventional high resolution electron microscopic analysis on Epon-embedded tissue revealed various degrees of morphological alterations. These morphogenic changes were equal in both transgenic lines and ranged from relatively subtle ultraterminal branches, originating from the synaptic bouton or the preterminal axon shaft and wrapping around synaptic core elements containing synaptic vesicles, to elaborate labyrinths of multiple layers of axolemma interspaced with ultrathin sheaths of axoplasm (Fig. 6). The most complex structures usually contained only a very small compressed core element devoid of synaptic vesicles and with occasional electron-dense inclusion bodies.

In the Ad-B-50/GAP-43-injected mice, the complexity of morphologically changed axon structures increased with time. At 3 d after infusion of Ad-B-50/GAP-43, we encountered synaptic profiles consisting of a synaptic core, synaptic vesicles, and an occasional synaptic density, and ultraterminal branches arising at or just proximal to the synapse (Fig. 7*A–C*). During the following days (5–12 d), these altered synaptic boutons developed in progressively complex axonal labyrinths (Fig. 7*D,E*). Synaptic core elements often were completely surrounded by multiple sheaths of membrane. In the core, electron-dense axoplasm, vesicles and

occasional mitochondria could still be seen, although less as compared with the 3 d time point. The axoplasm between the axolemmal sheaths was in continuity with the synaptic core. The sheaths, however, were not arranged in a spiral fashion, like Schwann cell processes during the formation of myelin sheaths, but were concentrically organized (Fig. 7*C*). Examination of glomeruli in Epon-embedded olfactory bulbs from mice infused with Ad-LacZ did not reveal structures with an altered membrane organization (data not shown). Olfactory bulbs from these mice, 12 d after infusion, were labeled for β -gal. β -Gal-positive primary olfactory axon endings exhibited a normal morphology and contained vesicles and normal synaptic contacts with dendrites (Fig. 7*F*), indicating that transduction with a control adenoviral vector does not result in alterations in synaptic morphology as observed after adenoviral vector-directed expression of B-50/GAP-43.

DISCUSSION

To investigate the role of the growth-associated protein B-50/GAP-43 *in vivo*, two distinct gene transfer technologies were used. First, we created an adenoviral vector to target B-50/GAP-43 to adult olfactory neurons. The use of an adenoviral vector permits the temporal dissection of effects of B-50/GAP-43 on the morphology of mature olfactory neurons. Second, we used B-50/GAP-43 transgenic mice with constitutive expression of B-50/GAP-43 in olfactory neurons. By confocal and electron microscopy, we show that expression of B-50/GAP-43 in adult primary olfactory neurons induces a state of growth at primary olfactory synapses, as evidenced by the formation of thin axonal extensions arising just proximal to or at the actual synaptic bouton. A temporal analysis of the morphological changes revealed an increasing complexity of olfactory nerve endings at longer intervals after B-50/GAP-43 expression via Ad-B-50/GAP-43, eventually resulting in axonal labyrinths predominantly at the glomerular boundary formed by juxtglomerular cells. These observations suggest that this neuronal growth cone protein can

Figure 5. Immunoelectron microscopical analysis of mature primary olfactory axon profiles: coexpression of OMP and B-50/GAP-43 in axonal labyrinths. Ultrathin sections of Lowicryl-embedded olfactory bulbs of transgenic mice were labeled for B-50/GAP-43 (A–D) and OMP (E). A–C, Axon profiles (indicated by two arrowheads in C) that terminate in large concentrically organized membrane structures are always highly labeled for B-50/GAP-43 and are found predominantly in the vicinity of the glomerular border delineated by juxtaglomerular cells (indicated by jc in B and C). In adjacent sections the ultrastructurally abnormal axon endings were immunolabeled for B-50/GAP-43 (D) and OMP (E), indicating that axonal labyrinth formation occurs in mature OMP-positive olfactory axons overexpressing B-50/GAP-43. Scale bar (shown in E): A, 3.8 μ m; B, 3.4 μ m; C–E, 2.6 μ m.

promote the expansion of neuronal plasma membrane at synaptic boutons of adult olfactory neurons.

Adenoviral vector-mediated gene transfer in mature olfactory neurons

Adenoviral vectors allow gene transfer to a wide range of postmitotic neural cells (Akli et al., 1993; Bajocchi et al., 1993; Davidson et al., 1993; Le Gal La Salle et al., 1993). This is a valuable approach because it is thus far the only method for obtaining overexpression of a particular gene product in a selected region of the nervous system of fully developed rodents. Efficient adenoviral vector-mediated expression of the reporter gene *LacZ* to mature, OMP-positive olfactory neurons has been documented previously (Holtmaat et al., 1996); however, at least two strict criteria have to be met to allow valid conclusions on the phenotypic effects of B-50/GAP-43 on primary olfactory neurons expressed via an adenoviral vector. First, gene transfer with an adenoviral vector should not affect the neuronal turnover process that occurs in this neuroepithelium throughout adult life. Second, infection of the olfactory neuroepithelium with an adenoviral vector should not result in induction of the expression of the endogenous *B-50/GAP-43* gene. The BrdU-labeling index of virus buffer-treated and adenoviral vector-treated olfactory neuroepithelium was not significantly different and was three times lower than the labeling index observed after toxic injury or lesions (Carr and Farbman, 1992; Schwob et al., 1995). Double-labeling of Ad-*LacZ*-transduced olfactory epithelium with β -gal and B-50/GAP-43 antibodies revealed the previ-

ously reported complement of B-50/GAP-43-positive cells restricted to the basal region of the olfactory neuroepithelium (Verhaagen et al., 1989) and no expression of B-50/GAP-43 in Ad-*LacZ*-transduced, β -gal-expressing, mature olfactory neurons in the upper portion of the neuroepithelium. These findings show that the olfactory neuroepithelium transduced with an adenoviral vector has a normal neuronal turnover and displays the anticipated expression of endogenous B-50/GAP-43 restricted to the basal cell region.

Expression of B-50/GAP-43 in mature olfactory neurons induces axonal labyrinths

Ad-*LacZ* and Ad-*B-50/GAP-43* efficiently transduced mature primary olfactory neurons, as indicated by double-labeling of β -gal or B-50/GAP-43 with OMP. At intervals ranging from 3 to 12 d after injection of Ad-*LacZ*, β -gal expression was observed in individual olfactory axons throughout individual glomeruli. The morphology of the control axons had a striking resemblance to previously shown Golgi-stained olfactory axon profiles (Halász and Greer, 1993; Holtmaat et al., 1995). The β -gal-positive olfactory fibers were often topped with small synaptic boutons that exhibited a normal ultrastructure, showing that adenovirus-based gene transfer does not induce morphological changes. The morphological changes in primary olfactory axons expressing B-50/GAP-43 reflect two processes: first, the induction of a growth state in synaptic boutons, and second, the formation of complex structures i.e., axonal labyrinths.

The initial morphological effects observed at 3 and 5 d after

Figure 6. Various degrees of ultrastructurally altered axon profiles in transgenic mice. Ultrathin sections of Epon-embedded olfactory bulbs of a wild-type mouse (*A*) and transgenic littermate of L29 (*B–E*). In Epon-embedded olfactory bulbs, primary olfactory axon terminals have a relative electron-dense axoplasm (indicated by the asterisk in *A*), whereas dendritic profiles (indicated by *d*) exhibit an electron lucent appearance. Various degrees of ultrastructural alterations are present in glomeruli of transgenic mice, ranging from subtle (*B*) and moderate (*C*) to elaborate (*D*) axonal labyrinths. Some axon profiles exhibit a few sheaths of axolemma interspaced with axoplasm. The synaptic core elements contain small numbers of vesicles (arrow in *C*) compared with primary olfactory axon endings in wild-type mice (*A*). The elaborate axonal labyrinths consist of multiple layers of axolemma (*E*, higher magnification of square area in *D*) interspaced with ultrathin sheaths of axoplasm (*a*) and extracellular components (*e*). Note that the axonal labyrinth (*D*) occurs in close approximation with a glial cell process (indicated by *gc*) or in the vicinity of juxtaglomerular cell bodies (indicated by *jc*). Scale bar (shown in *E*): *A–D*, 1.5 μm ; *E*, 0.02 μm .

Figure 7. Temporal dissection of morphological alterations in synaptic structures in Ad-B-50/GAP-43-infused mice. Ultrathin sections of Epon-embedded olfactory bulbs were taken from Ad-B-50/GAP-43- (A–E) or Ad-LacZ-injected (F) mice at 3 (A–C), 5 (D), and 12 (E, F) d after infusion of the viral vectors. A and B show examples of primary olfactory synapses that exhibit ultraterminal protrusions (arrows) arising from the synaptic bouton. C shows a synaptic bouton at 3 d with relatively advanced structural changes. Note that at 3 d the synaptic elements contain numerous vesicles and postsynaptic densities (indicated by *pd* in A). Furthermore, it can be clearly seen that the axolemmal extensions are continuous with the synaptic core (arrowhead in C). At 5 and 12 d, these structures have developed in axonal labyrinths with a synaptic core element virtually devoid of synaptic vesicles. Control axons expressing β -gal after transduction with Ad-LacZ are identified by preembedding labeling for β -gal. These axons exhibit a normal morphology. Scale bar (shown in F): A, D, E, F, 0.7 μ m; B, 0.35 μ m; C, 0.25 μ m.

viral vector-mediated expression of B-50/GAP-43 indicate that B-50/GAP-43 causes the formation of multiple thin extensions at or just proximal to the actual synaptic bouton. It has been proposed that B-50/GAP-43 expression in certain populations of synapses in the adult nervous system occurs in relation to structural plasticity (Benowitz et al., 1988; Neve et al., 1988). Evidence for a close association between elevated B-50/GAP-43 expression and sprouting of intact adult CNS neurons has been reported recently in the hippocampus after treatment with kainic acid (McNamara and Routtenberg, 1995; Cantallops and Routtenberg, 1996). In this paradigm, kainic acid is administered to activate limbic circuits in the hippocampus without inducing neural damage. Kainic acid-induced mossy fiber sprouting is preceded by upregulation of B-50/GAP-43 in granule cells, suggesting that B-50/GAP-43 is one of the proteins involved in this sprouting response. Transgenic mice overexpressing B-50/GAP-43 have extended mossy fiber projections, showing a direct link between B-50/GAP-43 and mossy fiber sprouting in the hippocampus (Aigner et al., 1995). Our results support and extend these findings by showing that a very brief period (3–5 d) of elevated B-50/GAP-43 expression in adult neurons can induce structural changes in synaptic boutons.

At 8 and 12 d, the formation of axonal labyrinths is indicative of the progressive addition of plasma membrane. The formation of axonal labyrinths has previously been noted in sensory projections in the dorsal horn of the rat and monkey spinal cord 2 weeks after transection of the sciatic nerve (Knyihár and Csillik, 1976; for review, see Csillik and Knyihár-Csillik, 1986). Peripheral sciatic nerve transection is normally followed by regeneration of the peripheral axons and enhances the regenerative capacity of the central axons of the dorsal root neurons when these are damaged simultaneously (Richardson and Issa, 1984). Peripheral injury to dorsal root ganglion neurons results in the initiation of the expression of a set of neuronal growth-associated proteins, including the upregulation of B-50/GAP-43 (Bisby, 1988; Verhaagen et al., 1988; Tetzlaff et al., 1989). Interestingly, B-50/GAP-43 is not only transported to the periphery but also accumulates in the central terminals of the axotomized dorsal root ganglion neurons (Woolf et al., 1990; Schreyer and Skene, 1991). The morphological changes in intact central projections after peripheral transection include the formation of compact axolemmal sheaths arising from ultraterminal branches (Knyihár and Csillik, 1976). The similarity between these morphological changes and the current morphological alterations in olfactory synapses expressing B-50/GAP-43 via a viral vector and in B-50/GAP-43-transgenic mice strongly suggest that B-50/GAP-43 is a critical permissive factor responsible for these morphological changes. By analogy, the formation of axonal labyrinths by central axons of peripherally axotomized dorsal root ganglion neurons (Knyihár and Csillik, 1976) appears to be an active process probably induced by elevated levels of B-50/GAP-43 in intact neuronal projections.

An issue that remains open is how B-50/GAP-43 might function to modify synaptic morphology. B-50/GAP-43 is located at the inside of the neuronal plasma membrane (Gorgels et al., 1989; Skene and Virag, 1989; Van Lookeren Campagne et al., 1989), acting in the context of other components of the synapse. Molecular interactions between B-50/GAP-43, calmodulin (Liu and Storm, 1990), the G-protein G_0 (Strittmatter et al., 1990, 1993), and the phosphorylation of B-50/GAP-43 by protein kinase-C (Aloyo et al., 1983; Zwiers et al., 1985) indicates that this GAP stands in the center of important signal transduction cascades at

the neuronal plasma membrane. In transgenic mice overexpressing nonphosphorylatable B-50/GAP-43, significantly less spontaneous and induced sprouting occur as compared with transgenic mice overexpressing the wild-type protein (Aigner et al., 1995). Studies on the interaction of B-50/GAP-43 with G_0 have revealed that B-50/GAP-43 increases the response to G-protein-coupled receptor agonists (Strittmatter et al., 1990, 1993) and may thereby enhance the sensitivity of a growth cone or synapse to signals in the neural environment (Igarashi et al., 1993, 1995; Strittmatter et al., 1994, 1995). The dynamic pattern of morphological alterations in olfactory axons indicates that B-50/GAP-43 promotes the growth of primary olfactory axons within the confines of the glomerulus but not into the deeper layers of the olfactory bulb. The glomerular neuropil provides a substrate permissive for axonal extension, because axons of newly formed olfactory neurons penetrate into the glomeruli throughout adulthood, and juvenile forms of cell-adhesion molecules and glycoproteins with a function in axon guidance and target recognition are continually expressed in the glomerular neuropil of adult mice (Miragall et al., 1988; Guthrie and Gall, 1991; Key and Akesson, 1991; Gonzalez et al., 1993). Thus, one explanation for the current findings may be that B-50/GAP-43 enhances the capacity of an olfactory axon terminal to display growth in response to intra-glomerular growth signals.

B-50/GAP-43-induced axonal labyrinths occur predominantly at the glomerular boundary

At longer intervals after B-50/GAP-43 overexpression, B-50/GAP-43-positive olfactory axon endings become visible at the rim of the glomeruli where they apparently cease to grow but continue to accumulate axolemma, resulting in extremely large axonal labyrinths. These axonal labyrinths could be derived from axons that have translocated their endings toward the glomerular edge or from axons that already projected into the periphery of the glomeruli. Axonal labyrinth formation appeared to occur predominantly in the vicinity of the glomerular boundary formed by juxtglomerular neurons, astrocytes, and oligodendrocytes (Valverde and Lopez-Mascaraque, 1991; Bailey and Shipley, 1993). Axonal growth into and beyond the juxtglomerular layer may be prevented by inhibitory molecules expressed by the juxtglomerular cells. Myelin-associated molecules (Caroni and Schwab, 1988; Schwab et al., 1993), several extracellular matrix molecules (Gonzalez et al., 1993), or the recently discovered chemorepulsive protein semaphorin(D)III/collapsin-1 (Luo et al., 1993; Messersmith et al., 1995; Puschel et al., 1995; Wright et al., 1995; Giger et al., 1996; Shepherd et al., 1996) are expressed by juxtglomerular cells and would be candidates to inhibit growth of transgenic fibers into the deeper layers of the bulb. In view of this, it is of interest that semaphorin(D)III/collapsin-1 is downregulated postnatally in most regions of the nervous system but continues to be expressed at high levels in the target cells (juxtglomerular and mitral cells) of primary olfactory neurons in the olfactory bulb during adulthood (Giger et al., 1996).

The present *in vivo* gene transfer study shows that the intrinsic growth cone protein B-50/GAP-43 triggers a state of growth in primary olfactory synapses. Neuronal membrane expansion at synaptic boutons overexpressing B-50/GAP-43 suggests that this protein is a critical determinant in ongoing structural synaptic plasticity in the adult nervous system and may have a role in local neuronal membrane repair after injury to synaptic projections.

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