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Cell-Specific Inducible Gene Recombination in Postnatal Inner Ear Supporting Cells and Glia

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ABSTRACT

Recent studies indicate that supporting cells play important roles in inner ear development, function, and regeneration after injury, but the molecular mechanisms underlying these processes remain poorly understood. Inducible cell-specific gene recombination in supporting cells could be a powerful tool to study the roles of specific molecules in these cells. Here we tested the feasibility, effectiveness, and cell specificity of inducible Cre-mediated gene recombination in the postnatal inner ear using mice that express an inducible form of Cre ($CreER^T$) under the transcriptional control of the proteolipid protein (PLP) promoter. We assessed the pattern of tamoxifen-induced gene recombination in the inner ear using the ROSA26-LacZ reporter line, in which the β-galactosidase gene is expressed only after Cre-mediated excision of a loxP-flanked stop cassette. Recombination was detected in cochlear inner phalangeal cells, supporting cells surrounding hair cells in vestibular maculae and cristae. Recombination also occurred in Schwann cells. We also found that this $CreER^T$ line can be used to increase and decrease the levels of expression of a trophic factor, brain-derived neurotrophic factor, specifically in supporting cells. These results show that PLP/$CreER^T$ mice are a powerful tool to dissect gene function in inner ear supporting cells.

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INTRODUCTION

Cochlear and vestibular sensory epithelia contain two primary cell types, hair cells and supporting cells. Although the roles of hair cells and their associated sensory neurons are well established, the functions of supporting cells are now beginning to emerge. There is increasing evidence that these non-neuronal cells play important roles in the development, maintenance, and function of the inner ear. For example, supporting cells act as precursors for hair cells during embryogenesis and can later trans-differentiate into hair cells in the adult avian inner ear (Morest and Cotanche 2004; Ryals and Rubel 1988). While adult mammalian supporting cells do not normally generate hair cells after injury, some studies indicate that they can do it if they are induced to express specific genes, such as the transcription factor Math1 (Gubbels et al. 2008). Thus, manipulation of supporting cell gene expression could one day become a tool to treat some forms of hearing loss.

Supporting cells also appear to be important regulators of synaptic activity in the sensory epithelia. Specifically, supporting cells of the organ of Corti contribute to spontaneous activity of the auditory nerve prior to the onset of hearing (Tritsch et al. 2007). In the mature sensory epithelia, glutamate transporters expressed by supporting cells have been implicated in the regulation of neurotransmission at the hair cell-afferent fiber synapse (Glowatzki et al. 2006). There is also evidence indicating that supporting

cells contribute to the long-term survival of type I spiral ganglion neurons (Stankovic et al. 2004; Sugawara et al. 2005). This promotion of neuronal survival appears to be mediated by supporting cell-derived neurotophic factors such as neurotrophin 3 (Stankovic et al. 2004).

Further understanding of the roles of supporting cells in the postnatal inner ear would be greatly facilitated by the use of genetically modified mice. However, since many of the genes of interest are critical to the development and function of other tissues and cell types, it would be crucial to find a way to temporally alter gene expression specifically in supporting cells. We decided to test if this could be achieved using transgenic mice expressing tamoxifen-inducible Cre recombinase. We identified a mouse line that was likely to express CreER^T in supporting cells under the control of the proteolipid protein (PLP) promoter (Doerflinger et al. 2003). While the PLP gene encodes for proteolipid protein known to be expressed by myelin-producing cells of the nervous system (Schwann cells and oligodendrocytes; Fuss et al. 2000; Mallon et al. 2002; Wight et al. 1993), a recent study showed that the PLP promoter is active in supporting cells of the inner ear at embryonic stages (Morris et al. 2006). Here we show that the PLP promoter continues to be active in supporting cells of the cochlea and vestibular organs at postnatal ages. Using the ROSA26-LacZ reporter mouse line, we found that tamoxifen treatment of PLP/CreER^T mice any time between birth and postnatal day 17 (P17) induces effective gene recombination in cochlear inner phalangeal cells, supporting cells surrounding hair cells in vestibular maculae and Schwann cells in the osseous spiral lamina. Furthermore, we also found that expression of the trophic factor brain-derived neurotrophic factor (BDNF) can either be knocked out or induced in the vestibular epithelia of mice at the same ages using this PLP/CreER^T line. These results show that PLP/CreER^T mice are a powerful tool to dissect gene function in inner ear supporting cells and glia.

MATERIALS AND METHODS

Animals

PLP/CreER^T (Doerflinger et al. 2003), BDNF$^{f/f}$ (Rios et al. 2001), and ROSA26-LacZ (Soriano 1999) mice were obtained from the Jackson Laboratory. Previously characterized mice expressing eGFP under the control of the PLP gene promoter were used to study the pattern of PLP-promoter activity in the postnatal inner ear (Mallon et al. 2002). The BDNF^{stop} mouse line (Chang et al. 2006) was provided by Dr. Rudolf Jaenisch, Whitehead Institute for Biomedical Research, Cambridge, MA, USA. Mice were maintained in the animal facility at Children's Hospital Boston, and all procedures were carried out following protocols approved by the Children's Hospital Animal Care and Use Committee. At least three animals (six ears) were evaluated for recombination. Age-matched, wild-type littermates were used as controls.

Visualization of GFP

Mice were anesthetized with 2.5% Avertin (0.2 ml/10 g body weight) and fixed by intracardial perfusion with 4% paraformaldehyde. Temporal bones were dissected, incubated in 30% sucrose, and frozen. Sections (10 μm) were cut by cryostat. For whole-mount images, cochleas, utricles, and cristae were dissected and immediately observed under the fluorescent microscope.

Tamoxifen injection

Tamoxifen (Sigma, St. Louis, MO, USA) was dissolved in corn oil (Sigma) at a concentration of 10 mg/ml at 55°C. A 30 G1/2 needle insulin syringe was used for intraperitoneal injections in pups. All different combinations of mice were injected for seven consecutive days, starting at postnatal day 0 (P0) P5 or P10 with tamoxifen at a dose of 33 mg/kg body weight or with the corn oil carrier alone.

LacZ (β-galactosidase) staining

Mice were anesthetized as described above and fixed by intracardial perfusion with 2% paraformaldehyde–0.2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The temporal bones were dissected and perfused by flushing the fixative solution through the oval window. The temporal bones were then washed three times for 30 min at room temperature and incubated in staining solution for 6 h at 37°C in the dark. The staining solution contained 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3\ H_2O$, 2 mM $MgCl_2.6\ H_2O$, 0.01% Na-deoxycholate, 0.02% NP-40, and 1 mg/ml X-gal. Tissues were rinsed three times with PBS for 5 min, postfixed overnight with the same fixing solution as above, washed with PBS, and decalcified for 2–3 days in 4% EDTA. Then, the tissues were embedded in araldite by using a rapid dehydration protocol to minimize washout of reaction product. No differences in the intensity or pattern of lacZ staining were observed before and after dehydration. Araldite-embedded materials were sectioned at 20 μm on a microtome (Leica). Ears of PLP/CreER^T::ROSA26-LacZ double-transgenic mice injected with vehicle alone were processed in parallel as control.

Immunohistochemistry

Mice were anesthetized with 2.5% Avertin (0.2 ml/10 g body weight). The temporal bones were dissected, and the utricular maculae were removed and fixed in methanol at 4°C for 20 min as described in Montcouquiol et al. (1998). Tissues were washed in phosphate buffer and blocked for 2 h in 10% normal goat serum, 0.1% Triton-X, and 0.2% Tween. Chicken anti-BDNF (1:50, Promega, Madison, WI, USA) was incubated overnight at 4°C. Tissues were then washed in phosphate buffer and followed by overnight incubation with goat anti-chicken Alexa-488 (1:300, Molecular Probes, Eugene, OR, USA) at 4°C.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed as in Stankovic and Corfas (2003). Briefly, P21 mice were killed, inner ears were extracted, cochlear and vestibular organs were dissected from otic capsule in RNAlater (Ambion, Austin, TX, USA), and total RNA was purified using RNeasy spin-columns (Qiagen, Valencia, CA, USA). Two inner ears from a single animal were processed as one sample. Only RNA samples that fulfilled the criteria that were established previously (Stankovic and Corfas 2003) were used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Samples without reverse transcriptase were processed in parallel and served as negative controls. Measurements were made with an iCycler machine (Bio-Rad) and IQ SYBR Green supermix kit (Bio-Rad). For each well of the 96-well plate (Bio-Rad), the 25 µl reaction contained 12.5 µl of 2X IQ SYBR Green supermix, 0.5 µM each forward and reverse primer, 7.5 µl of RNase-free H_2O, and 2.5 µl cDNA template. The cycling conditions were as follows: 95°C for 15 min followed by 50 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 60 s. Each sample was loaded in triplicate. The following forward (F) and reverse (R) primers were used: for 18S rRNA (gene accession number X00686), F: CGGCTACCA CATCCAAGGAA, R: GCTGGAATTACCGCGGCT (Hellstrom et al. 2001), which generates a 187-bp amplicon. For BDNF (gene accession number NM_007540), F: GTGTGTGACAG TATTAGCGAGTGG, R: GATACCGGGACTTTCTC TAGGAC, which generates a 101-bp amplicon.

RESULTS

Pattern of PLP-promoter activity in the postnatal inner ear

A recent study showed that the PLP promoter drives eGFP expression in supporting cells of the embryonic cochlea (Morris et al. 2006). Since other cells that express PLP during development continue to express relatively high levels of this molecule in the adult, e.g., oligodendrocytes (Mallon et al. 2002), we hypothesized that the same would be true for inner ear supporting cells. To test this possibility and to expand the analysis of PLP-promoter activity to the vestibular system, we examined the pattern of eGFP expression in the inner ear of PLP-eGFP mice at different postnatal ages (P3, P5, P15, and P21). Since results were similar at all ages, only P15 images are shown. In the organ of Corti, the only supporting cells that are positive for eGFP are those that surround the IHCs, i.e., the inner phalangeal cells, their fluorescence being intense without any obvious apex-to-base gradient. Schwann cells in the osseous spiral lamina also express eGFP (Fig. 1A, B). In the utricle and saccule, most supporting cells abutting hair cells also express eGFP, although the intensity of labeling varies among them (Fig. 1C, D). Similar to the organ of Corti, vestibular Schwann cells are also eGFP positive (Fig. 1D). In contrast to the maculae, not all supporting cells express eGFP in the cristae ampullaris (Fig. 1E, F). Rather, eGFP expression is restricted to supporting cells located in the base of the cristae.

Using a reporter line to characterize the pattern of tamoxifen-induced recombination in the inner ear of postnatal PLP/CreER^T mice

To directly test the ability of the PLP promoter to drive inducible gene recombination in postnatal inner ear supporting cells, we obtained a transgenic line in which the tamoxifen-inducible Cre (CreER^T) is under the control of the PLP promoter (Doerflinger et al. 2003) and crossed it with the ROSA26-LacZ reporter mouse line in which the LacZ gene encoding β-galactosidase (β-gal) is expressed only after the Cre-mediated excision of a loxP-flanked stop cassette (Soriano 1999). PLP/CreER^T::ROSA26-lacZ mice were injected intraperitoneally with either vehicle or tamoxifen at a dose of 33 mg/kg body weight daily for seven consecutive days beginning at P10. Temporal bones were collected 1 day after the last injection (P17), processed for β-gal histochemistry, and embedded in plastic. Examination of temporal bone whole mounts showed that tamoxifen induced effective recombination in the inner ear of PLP/CreER^T::ROSA26-LacZ double-transgenic mice (Fig. 2A). LacZ staining was intense in the sensory epithelia of the cochlea without any obvious apex-to-base gradient. Strong staining was also evident in the sensory epithelia of the vestibular organs. Importantly, no staining was present when double-transgenic mice were injected with vehicle alone, indicating that recombination occurred only upon tamoxifen injection (Fig. 2B). These results show that tamoxifen can

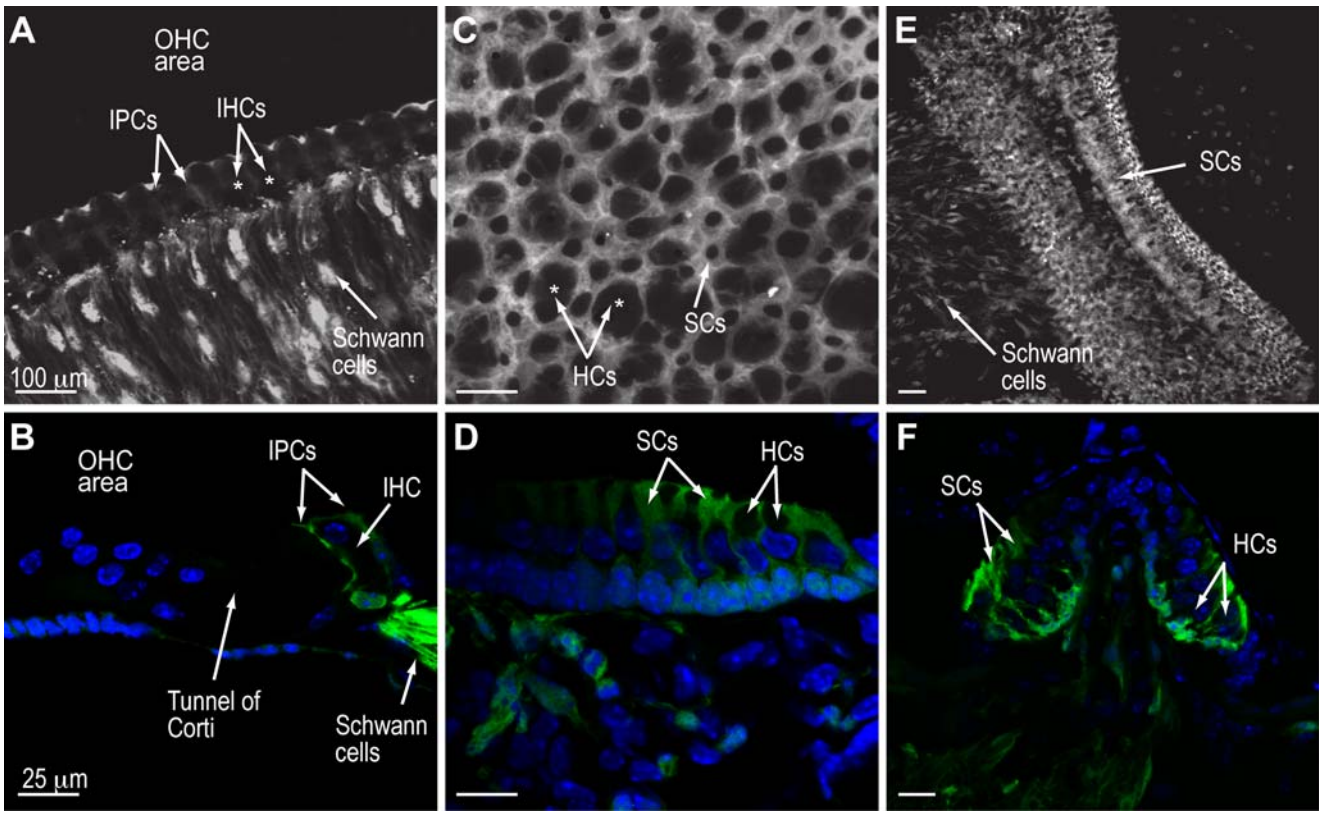

FIG. 1. PLP promoter activity in the postnatal inner ear. Bird's eye view of whole mounts (**A**, **C**, and **E**) and images of cross sections (**B**, **D**, and **F**) of organ of Corti (**A**, **B**), utricular macula (**C**, **D**), and cristae ampullaris (**E**, **F**) from P15 PLP-eGFP mice (*top* imaging of eGFP, *bottom* imaging of eGFP (*green*) and nuclear staining (*blue*)). The images show that the PLP promoter drives transgene expression in supporting cells (*SCs*) that surround inner hair cells (*IHCs*) and Schwann cells in the cochlea (**A**, **B**), supporting cells abutting hair cells (*HCs*) in the vestibular maculae (**C**, **D**) and in some supporting cells in the cristae (**E**, **F**). Vestibular Schwann cells also express eGFP (**D**, **E**, and **F**). *OHC* outer hair cell, *IPCs* inner phalangeal cells. *Scale bars*=100 μm (**A**, **C**, and **E**) and 25 μm (**B**, **D**, and **F**).

efficiently cross the barrier that exists between the blood circulation of the inner ear and the endolymph surrounding hair cells to effectively induce CreER^T activity in the inner ear.

To investigate the cell types in which gene recombination occurred—i.e., to identify the cells expressing the β-gal—the plastic-embedded whole mounts were sectioned and analyzed at higher magnification

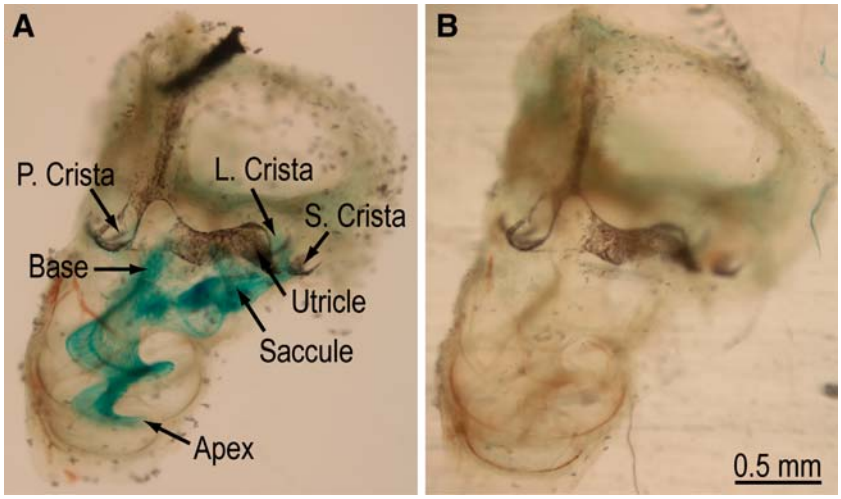

FIG. 2. Visualization of PLP/CreER^T-induced recombination using ROSA26-LacZ reporter mouse. **A** β-Galactosidase histochemistry in a temporal bone whole-mount from a PLP/CreER^T::ROSA26-LacZ double-transgenic mouse injected with tamoxifen from P10 to P16 and harvested at P17. **B** Whole-mount staining from a PLP/CreER^T::ROSA26-LacZ double-transgenic mouse injected with vehicle alone. Key structures are identified in **A**. Cochlear apex and base are indicated. Vestibular organs are labeled, including the cristae of the three semicircular canals (posterior [*P*], lateral [*L*], and superior [*S*]) and the maculae of the utricle and saccule. *Scale bar*=0.5 mm.

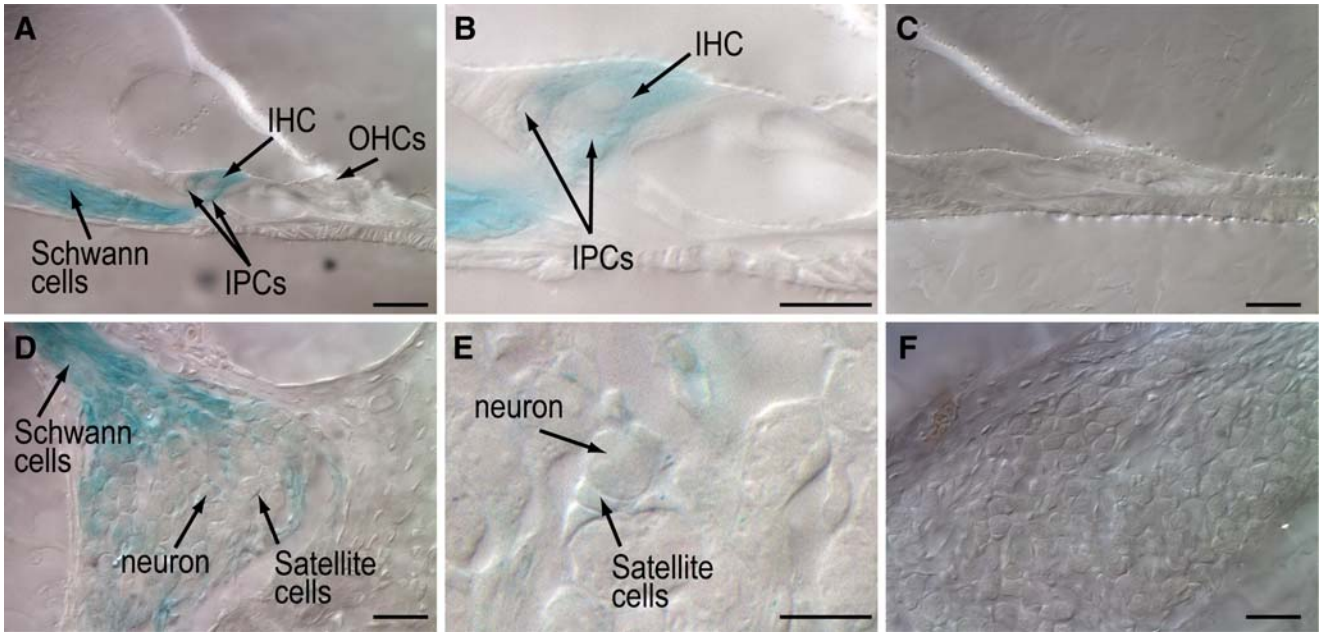

FIG. 3. PLP/CreER^T-induced gene recombination in the cochlea. β-Galactosidase (*β-gal*) histochemistry as seen in plastic sections (20 μm) through the organ of Corti (**A**, **B**) and spiral ganglion (**D**, **E**) from a P17 PLP/CreER^T::ROSA26-LacZ double-transgenic mouse injected with tamoxifen from P10 to P16. Images were taken from the basal turn, corresponding to a frequency of roughly 25 kHz (Sugawara et al. 2005). **C** and **F** show that β-gal staining is absent from PLP/CreER^T::ROSA26-LacZ double-transgenic mouse injected with vehicle alone. *IHC* inner hair cell, *OHCs* outer hair cells, *IPCs* inner phalangeal cells. *Scale bar*=25 μm in **A**, **C**, **D**, and **F**; 50 μm in **B** and **E**.

using established morphological criteria to identify the different cell types (Pannese 1981; Rio et al. 2002). In the organ of Corti, specific β-gal activity was detected in inner phalangeal cells adjacent to IHCs along the entire length of the cochlea (Fig. 3A, B). No signal was detected in hair cells or other supporting cell populations of the inner or outer hair cell areas. β-Gal was also expressed in Schwann cells in the osseous spiral lamina. In the spiral ganglion, no signal was apparent in the sensory neurons nor in the satellite cells that surround them (Pannese 1981; Fig. 3A, B, D, E). Double-transgenic mice injected with vehicle alone did not show evidence of recombination (Fig. 3C, F).

Tamoxifen-induced Cre activity was also present in the vestibular end organs. In the utricular maculae, recombination was robust in most supporting cells (Fig. 4A). Also, in the saccular maculae we observed recombination in most supporting cells surrounding hair cells (not shown). In contrast, recombination in the cristae ampullaris was less reliable, occurring only in a fraction of the supporting cells (Fig. 4B), and in some cases β-gal activity was undetectable in these organs (not shown). As in the cochlea, recombination was detected in vestibular Schwann cells (Fig. 4A, B) and was absent from vestibular sensory neurons (Fig. 4C). However, unlike the cochlea, recombination was strong in vestibular satellite cells (Fig. 4C). No β-gal activity was detected in the vestibular system of PLP/CreER^T::ROSA26-LacZ double-transgenic mice injected with vehicle alone (Fig. 4D–F). To study the pattern of tamoxifen-induced recombination at early time points, PLP/CreER^T::ROSA26-lacZ mice were injected daily for seven consecutive days beginning at P0 and P3. Temporal bones were collected 1 day after the last injection and processed for β-gal histochemistry as described above. We observed that gene recombination occurred in the same cell types as mice that were injected beginning at P10 (data not shown).

Tamoxifen induces effective recombination of the BDNF locus in the inner ear of postnatal PLP/CreER^T mice

The evidence provided by the reporter mice indicated that the PLP/CreER^T mice could be used to increase or decrease the expression of molecules of interest in supporting cells and some glia in the postnatal inner ear. We decided to test this by focusing on the neurotrophin BDNF, a molecule that has been shown to play important roles in the inner ear during embryonic stages (Ernfors et al. 1995; Ernfors et al. 1994) but whose function or expression pattern in the postnatal ear has not been defined. To test our ability to knockdown BDNF expression we crossed PLP/CreER^T mice with mice in which the BDNF gene has been flanked by loxP sites (BDNF$^{f/f}$; Rios et al. 2001). PLP/CreER^T::BDNF$^{f/f}$ and BDNF$^{f/f}$ mice were injected with tamoxifen for seven consecutive days (P5–P11). When the animals reached P21, inner ears were

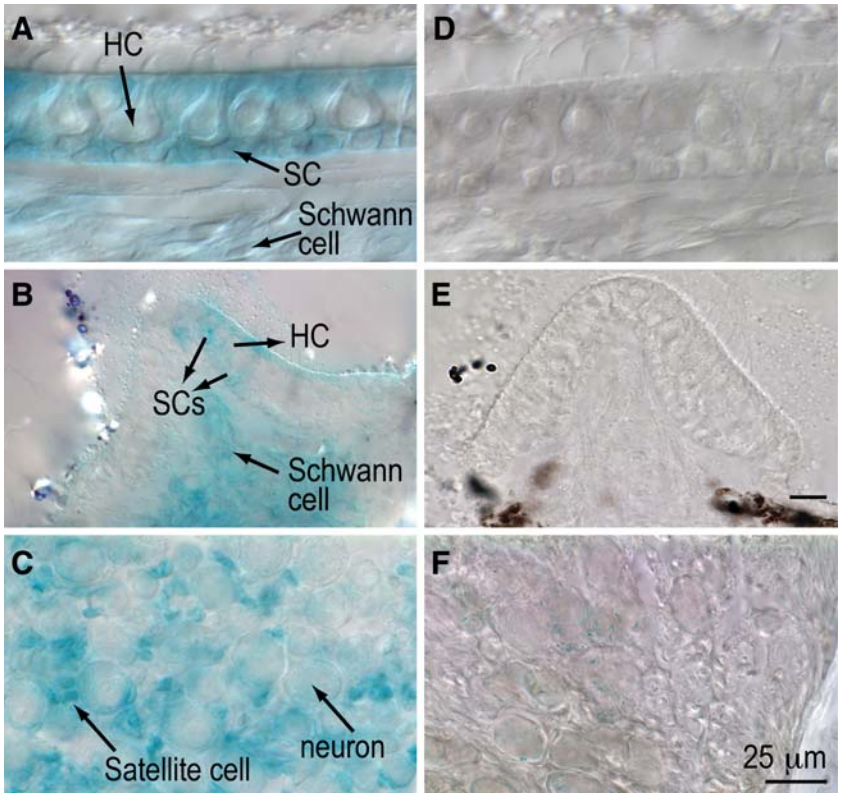

FIG. 4. PLP/CreER^T-induced gene recombination in the vestibular organs. β-Galactosidase histochemistry as seen in plastic sections (20 μm) of the utricle (**A**), ampulla of the posterior canal (**B**), and vestibular ganglia (**C**) from a P17 PLP/CreER^T::ROSA26-LacZ double-transgenic mouse injected with tamoxifen from P10 to P16. **D**, **E**, and **F** show that β-gal staining is absent from the utricle (**D**), ampulla of the posterior canal (**E**), and vestibular ganglia (**F**) from PLP/CreER^T:: ROSA26-LacZ double-transgenic mouse at P17 injected with vehicle alone. *HC* hair cell, *SC* supporting cell. *Scale bar*=25 μm.

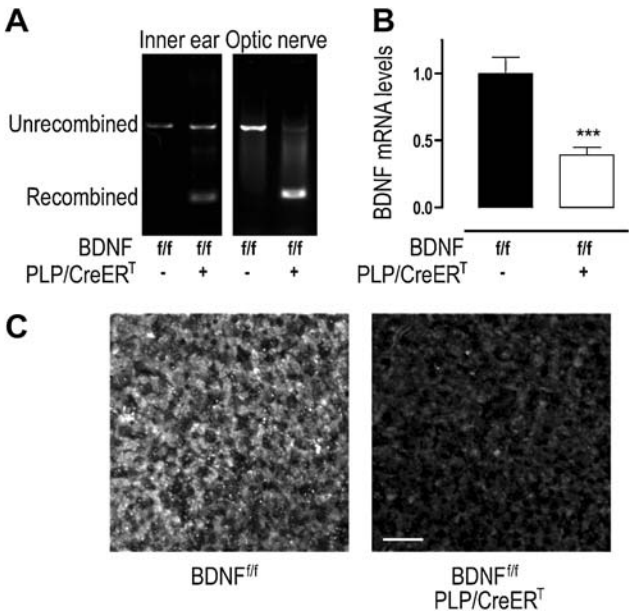

FIG. 5. The PLP/CreER^T transgene allows for conditional BDNF knockdown in the postnatal inner ear. **A** Tamoxifen induces recombination of the BDNF gene in the inner ear of PLP/CreER^T:: BDNF$^{f/f}$ mice. PCR of inner ear and optic nerve genomic DNA extracts with primers that produce different bands for the unrecombined (1 kb) and recombined (0.25 kb) BDNF alleles. No recombination is observed in animals that do not carry the PLP/CreER^T transgene. **B** Real-time quantitative RT-PCR of whole inner ear RNA shows that tamoxifen induces knockdown of BDNF expression in the inner ear of mice carrying the PLP/CreER^T transgene and two copies of the BDNF^f allele (BDNF$^{f/f}$). **C** Immunostaining with BDNF antibodies of the utricular maculae shows that tamoxifen induces a reduction of BDNF expression in supporting cells of mice carrying the PLP/CreER^T transgene and two copies of the BDNF^f allele (BDNF$^{f/f}$).

dissected and analyzed. First, genomic DNA was purified and subjected to PCR with primers to test for recombination in the BDNF locus. Optic nerves were processed in parallel as positive controls since the nerve is primarily comprised of PLP-expressing oligodendrocytes. As shown in Figure 5A, recombination of the BDNF locus in the optic nerve was almost complete. In this tissue, the unrecombined BDNF locus most probably represents astrocytes, endothelial and connective tissue cells, which do not express PLP. In the inner ear, the level of recombination was significant, although lower than in the optic nerve, consistent with the lower percentage of PLP-expressing cells. No recombination was observed in animals that did not carry the PLP/CreER^T transgene. Consistent with the apparent recombination of the BDNF gene, quantitative real-time PCR of inner ear extracts showed a dramatic reduction in the expression of BDNF in PLP/CreER^T::BDNF$^{f/f}$ mice after tamoxifen injection (Fig. 5B). Similarly, semi-quantitative immunostaining of BDNF in P21 utricular maculae showed that the levels of BDNF protein were lower in tissues from PLP/CreER^T::BDNF$^{f/f}$ mice after tamoxifen injection (Fig. 5C). In both cases, mRNA and protein, there was some residual BDNF signal in the vestibular system of PLP/CreER^T::BDNF$^{f/f}$ mice, most likely a reflection of incomplete knockdown. The immunostaining also showed that at this age BDNF expression is uniformly restricted to vestibular supporting cells and absent from hair cells, as shown previously by (Montcouquiol et al. 1998). Remarkably, the inner ears of mice in which BDNF was knocked down after P5 appeared normal under gross histological analysis, i.e., in size and general cellular organization (data not shown).

To test if the levels of BDNF expression in supporting cells and Schwann cells can be increased in the postnatal inner ear we used a mouse line with an inducible BDNF transgene (BDNF^{stop}; Chang et al. 2006). BDNF^{stop}::PLP/CreER^T and PLP/CreER^T mice were injected with tamoxifen for seven consecutive days starting at P5, and BDNF expression levels were examined at P21. Quantitative real-time PCR of inner ears showed that tamoxifen induced a 2.5-fold increase in BDNF expression in the double-transgenic mice (Fig. 6A). Semi-quantitative analysis of BDNF immunostaining in the utricular maculae indicated that the levels of BDNF protein were higher in tissues from mice that carry the PLP/CreER^T transgene in addition to the BDNF^{stop} transgene (Fig. 6B).

DISCUSSION

Our results show that the PLP/CreER^T mouse line represents a powerful tool to test the roles of supporting cells in the auditory and vestibular systems and the molecules involved in those processes. This mouse line will enable the manipulation of different classes of molecules in supporting cells, including trophic/growth factors or their receptors, transcription factors, cytoskeletal elements, and molecules involved in neurotransmitter function. Similarly, conditional manipulation of transcription factors involved in the genesis of hair cells by supporting cells could provide tests for the potential of hair cell regeneration in the adult (Gubbels et al. 2008). This Cre line, together with others that allow for inducible gene recombination in hair cells (Chow et al. 2006), provide unique opportunities to explore the cellular and molecular mechanisms that govern the inner ear and the pathogenesis of sensorineural hearing and balance disorders.

Our findings also provide another example of the analogies between supporting cells of the inner ear and glial cells. It is becoming clear that supporting cells have many similarities to glial cells, including the expression of numerous proteins, i.e., vimentin (Anniko et al. 1986), S100β (Pack and Slepecky 1995), glutamate-aspartate transporter (Furness and Lehre 1997; Glowatzki et al. 2006), low-affinity neurotrophin receptor p75 (Vega et al. 1999), the glial fibrillary acidic protein (GFAP; Rio et al. 2002), and

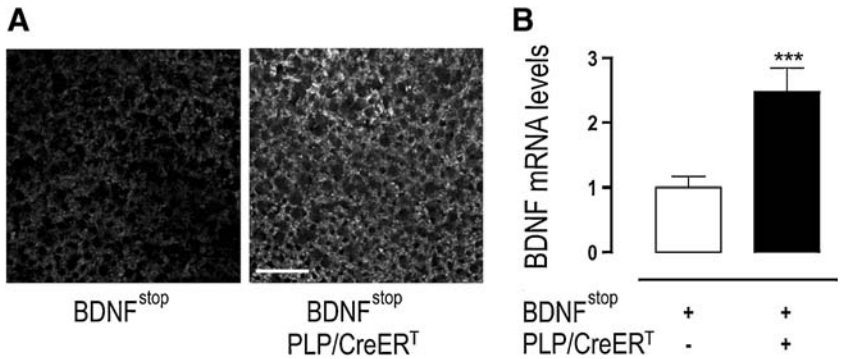

FIG. 6. The PLP/CreER^T transgene allows for conditional BDNF over-expression in postnatal supporting cells. Immunostaining with BDNF antibodies (**A**) and Real-time quantitative RT-PCR of whole inner ear RNA (**B**) show that tamoxifen increases BDNF expression in the utricular macula of mice carrying an inducible BDNF transgene (BDNF^{stop}) when mice carry the PLP/CreER^T transgene.

the PLP (Morris et al. 2006). In addition, our present work has expanded on the results of Morris et al. (2006), indicating that the PLP promoter is not only active in embryonic supporting cells but it also continues to be active in mature supporting cells. Interestingly, our analysis shows that PLP is not expressed by all supporting cells, i.e., in the cochlea is only expressed by supporting cells surrounding IHCs. It is possible that other $CreER^T$ lines such as those using the GFAP promoter (Hirrlinger et al. 2006) may serve as drivers to other supporting cells, in which we have shown this promoter to be active (Rio et al. 2002).

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