

# Caveolin-1 and Altered Neuregulin Signaling Contribute to the Pathophysiological Progression of Diabetic Peripheral Neuropathy

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**OBJECTIVE**—Evaluate if Erb B2 activation and the loss of caveolin-1 (Cav1) contribute to the pathophysiological progression of diabetic peripheral neuropathy (DPN).

**RESEARCH DESIGN AND METHODS**—Cav1 knockout and wild-type C57BL/6 mice were rendered diabetic with streptozotocin, and changes in motor nerve conduction velocity (MNCV), mechanical and thermal hypoalgesia, Erb B2 phosphorylation (pErb B2), and epidermal nerve fiber density were assessed. The contribution of Erb B2 to DPN was assessed using the Erb B2 inhibitors PKI 166 and erlotinib and a conditional bitransgenic mouse that expressed a constitutively active form of Erb B2 in myelinated Schwann cells (SCs).

**RESULTS**—Diabetic mice exhibited decreased MNCV and mechanical and thermal sensitivity, but the extent of these deficits was more severe in diabetic Cav1 knockout mice. Diabetes increased pErb B2 levels in both genotypes, but the absence of Cav1 correlated with a greater increase in pErb B2. Erb B2 activation contributed to the mechanical hypoalgesia and MNCV deficits in both diabetic genotypes because treatment with erlotinib or PKI 166 improved these indexes of DPN. Similarly, induction of a constitutively active Erb B2 in myelinated SCs was sufficient to decrease MNCV and induce a mechanical hypoalgesia in the absence of diabetes.

**CONCLUSIONS**—Increased Erb B2 activity contributes to specific indexes of DPN, and Cav1 may be an endogenous regulator of Erb B2 signaling. Altered Erb B2 signaling is a novel mechanism that contributes to SC dysfunction in diabetes, and inhibiting Erb B2 may ameliorate deficits of tactile sensitivity in DPN. *Diabetes* 58:2677–2686, 2009

**D**iabetic peripheral neuropathy (DPN) is a common complication of diabetes (1). Although hyperglycemia is the definitive cause of DPN (2), the vascular, glial, and neuronal damage that underlies the progressive axonopathy in DPN has a complex biochemical etiology involving oxidative stress (3,4), protein glycation (5), protein kinase C activation (6), polyol synthesis (7), and the hexosamine pathway (8). Altered neurotrophic support also contributes to sensory neuron dysfunction in DPN (9), but whether diabetes may alter growth factor signaling in Schwann cells (SCs),

which also undergo substantial degeneration in diabetes, is poorly defined.

Neuregulins are growth factors that control SC growth, survival, and differentiation via their interaction with Erb B receptors (10). Although Erb B2 signaling promotes developmental myelination and is clearly trophic for SCs, pharmacological evidence supports that pathologic activation of Erb B2 after axotomy (11) or infection with leprosy bacilli (12) is sufficient to induce SC dedifferentiation and demyelination. Additionally, genetic evidence supports that Erb B2 can promote the development of sensory neuropathies independent of diabetes because expression of a dominant-negative Erb B4 in nonmyelinating (13) or myelinating (14) SCs induced a temperature or mechanical sensory neuropathy, respectively. Given the contribution of Erb B2 to the degeneration of SCs, endogenous proteins that regulate Erb B2 activity may influence the development of certain aspects of sensory neuropathies.

The interaction of Erb B2 with the protein caveolin-1 (Cav1) inhibits the intrinsic tyrosine kinase activity of the receptor (15). Cav1 is highly expressed in mature, myelinated SCs (16), and we have shown that prolonged hyperglycemia promoted the downregulation of Cav1 in SCs of sciatic nerve (17). Cav1 may regulate Erb B2 signaling in SCs because its forced downregulation was sufficient to enhance neuregulin-induced demyelination of SC–dorsal root ganglion (DRG) neuron cocultures (18). However, it is unknown whether an increase in Erb B2 activity may contribute to the pathophysiological development of DPN and if changes in Cav1 expression may alter Erb B2 activation in diabetic nerve.

In the current study, we demonstrate that diabetic Cav1 knockout mice showed an increased activation of Erb B2 and developed greater motor nerve conduction velocity (MNCV) deficits relative to their wild-type counterparts. Inhibition of Erb B2 with two structurally diverse inhibitors corrected the MNCV deficits and mechanical hypoalgesia evident after 6 or 15 weeks of diabetes. Also, induction of a constitutively active Erb B2 in myelinated SCs of adult mice was sufficient to recapitulate the MNCV and mechanical sensitivity deficits observed in the diabetic mice. These studies provide the first evidence that activation of Erb B2 contributes to deficits associated with myelinated fiber function in diabetic nerve and suggest that Cav1 may serve as an endogenous regulator of Erb B2.

## RESEARCH DESIGN AND METHODS

Streptozotocin (STZ) was obtained from Sigma-Aldrich (St. Louis, MO). 4-(*R*)-phenethylamino-6-(hydroxyl) phenyl-7H-pyrrolo[2,3-*d*]-pyrimidine (PKI 166) and *N*-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine (erlotinib) were provided by Novartis Institutes for Biomedical Research (Basel, Switzerland) and OSI Pharmaceuticals (Melville, NY), respectively. The antibodies used and their sources were: Cav1 2234 (Transduction Labs, Lexington, KY); Erb B2 (Millipore, Billerica, MA); phospho-Tyr 1248 Erb B2 (pErb B2),

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TABLE 1  
FBG and weights of wild-type and Cav1 knockout mice

Week	FBG (mg/dl)	Weight (g)	n	FBG (mg/dl)	Weight (g)	n
Wild-type control				Wild-type STZ		
1	141 ± 17	21.1 ± 2.0	6	435 ± 71*	20.4 ± 1.6	7
2	154 ± 20	23.1 ± 2.0	9	563 ± 51*	20.5 ± 2.1	10
3	139 ± 23	22.1 ± 2.2	9	531 ± 83*	21.2 ± 1.8	8
4	142 ± 26	22.5 ± 1.68	10	508 ± 74*	21.5 ± 1.6	10
6	175 ± 26	27.5 ± 2.3	10	471 ± 81*	21.7 ± 2.7*	12
12	141 ± 21	25.8 ± 3.5	6	512 ± 113*	23.6 ± 2.18	7
Cav1 knockout control				Cav1 knockout STZ		
1	108 ± 18	22.5 ± 2.9	8	495 ± 94*	19.6 ± 1.7	8
2	140 ± 23	21.8 ± 2.8	8	521 ± 92*	18.8 ± 2.2	10
3	123 ± 26	22.5 ± 2.4	9	552 ± 60*	18.7 ± 3.2	11
4	112 ± 20	23.2 ± 3.0	8	564 ± 71*	22.4 ± 3.7	12
6	127 ± 13	25.5 ± 2.4	9	519 ± 106*	19.3 ± 3.3*	9
12	179 ± 31	32.2 ± 1.1	6	594 ± 11*	21.2 ± 3.1*	4

\* $P < 0.05$  vs. time-matched genotype control.

$\beta$ -actin, neurofilament H, and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); and AlexaFluor 488 rabbit anti-mouse and AlexaFluor 568 goat anti-rabbit antibodies (Molecular Probes, Eugene, OR).

**Animals and genotype analysis.** Wild-type C57Bl/6 and Cav1 knockout mice were obtained from Harlan Laboratories (Indianapolis, IN) and Jackson Labs (Bar Harbor, ME), respectively. The absence of Cav1 was confirmed by genotyping (19) and immunoblot analysis of lung and sciatic nerve (see supplemental Fig. 1, available in an online appendix at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-0594/DC1>). A conditional transgenic mouse based upon the tetracycline binary transgene system (20) was generated as described in the supplemental RESEARCH DESIGN AND METHODS. Briefly, transgenic mice that broadly express a constitutively active Erb B2 (caErb B2, V664-E664 mutation) under control of a minimal cytomegalovirus promoter and tetracycline response element (TRE) (21) were crossed with a separate transgenic line that expresses the reverse tetracycline transactivator (rtTA) gene under the control of the rat protein zero ( $P_0$ ) promoter. This strategy minimizes expression of the transgene in nonmyelinating SCs because  $P_0$  is a compact myelin protein (22). The caErb B2 transgene was induced by permitting ad libitum access to standard rat chow containing 2 g/kg doxycycline (Bio-Serv, Frenchtown, NJ).

**Induction of diabetes.** Eight-week-old wild-type and Cav1 knockout mice were rendered diabetic with three daily (75, 60, and 45 mg/kg), 0.2 ml i.p. injections of freshly prepared STZ (23). Three days after the last injection, mice with a fasting blood glucose (FBG) > 290 mg/dl (16 mmol/l) were deemed diabetic. All animals were maintained on a 12-h light/dark cycle with ad libitum access to water and Purina diet 5001 rodent chow. FBG levels were determined again immediately before euthanizing the animals. To inhibit Erb B2, biweekly intraperitoneal injections of 25 mg/kg PKI 166 in 10% DMSO or erlotinib in 6% Captisol were given. All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee and in compliance with standards and regulations for care and use of laboratory rodents set by the National Institutes of Health.

**Measure of mechanical and thermal sensitivity.** Mechanical sensitivity was assessed using a Dynamic Plantar Aesthesiometer (Stoelting, Wood Dale, IL) fitted with a stiff, 0.5-mm diameter monofilament that was delivered at an upward force of 8 g at a ramp speed of 2 s. The force (in grams) eliciting paw withdrawal was automatically recorded, and three to four responses taken on alternate feet were averaged.

Response to thermal stimuli was assessed using a Hargreaves Analgesiometer (24). Activation of the heat source projected a ramping focal radiant heat at the rate of about 0.3°C/s. Withdrawal latencies (in seconds) are the average of three to four trials taken from alternating feet with 5-min periods between testing.

**Assessment of nerve conduction velocity.** Mice were anesthetized, and body temperature was monitored with a rectal probe and maintained at 37°C using a heating pad connected to a Physitemp TCAT-2DF Controller (Physitemp Instruments, Clifton, NJ). Limb temperature was monitored with a subcutaneous sensor and maintained at 36–37°C with the aid of a heat lamp. The sciatic nerve was stimulated proximally at the sciatic notch and distally at the ankle via bipolar electrodes with a supramaximal stimulus (9.9 mA) of 0.05 ms duration with low and high filter settings of 3 and 10 kHz. MNCV (in meters/second) was calculated by measuring the latencies from the stimulus

artifact to the onset of the negative M-wave deflection of the compound muscle action potentials recorded from the first interosseous muscle and dividing by the distance (in millimeters) between the electrodes. For hindlimb sensory nerve conduction velocity (SNCV), the digital nerve to the second toe was stimulated with a square-wave pulse of 0.05-ms duration using the smallest intensity current that resulted in a maximal amplitude response, typically 2.4–3.0 mA. The sensory nerve action potential was recorded behind the medial malleolus, and the maximal SNCV was calculated by measuring the latency to the onset/peak of the initial negative deflection divided by the distance between stimulating and recording electrodes.

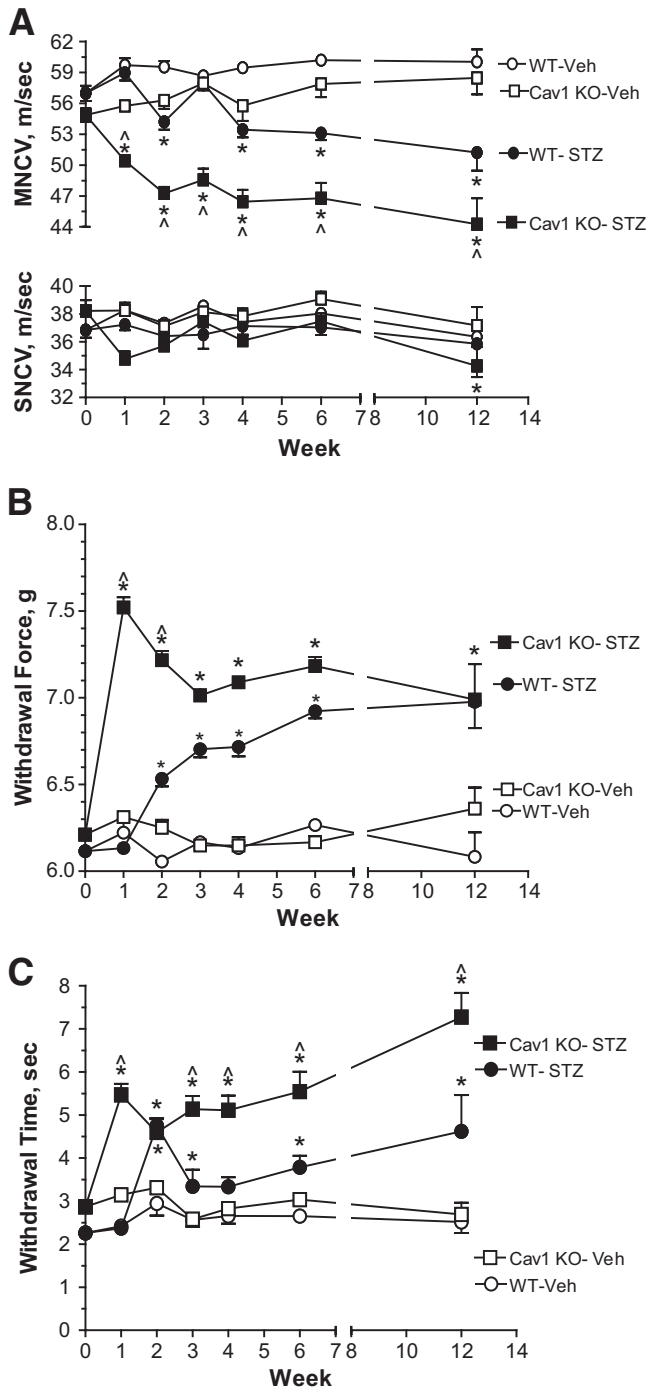
**Immunoblot and immunofluorescence analysis.** Nerves were homogenized in 0.2-ml lysis buffer (50 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 150 mmol/l  $\text{Na}_3\text{VO}_4$ , 0.5 mmol/l sodium molybdate, 40 mmol/l NaF, 10 mmol/l  $\beta$ -glycerophosphate, and 1X Complete Protease inhibitors) (Roche Diagnostics) with the aid of a Polytron fitted with a micro tissue tearor. Cell debris was sedimented at 10,000g for 5 min, and protein concentration of the supernatant was determined. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose for immunoblot analyses. Immunoblots were quantified by densitometry with the aid of ImageJ software and Erb B2 and pErb B2 levels were normalized to  $\beta$ -actin. Diabetes-induced changes in Erb B2 or pErb B2 were expressed as a percent of the control values. Immunofluorescence of sciatic nerve cross sections was performed as described (17).

**Statistical analyses.** Data are presented as means  $\pm$  SE. After verifying equality of variance, differences between treatments and genotypes were determined using a one-way or two-way ANOVA. Differences between group means were ascertained using Tukey test.

## RESULTS

**Absence of Cav1 enhances some of the phenotypic aspects of DPN.** Diabetes resulted in a three- to fourfold increase in FBG in both wild-type and Cav1 knockout mice (Table 1). Although both genotypes lost weight with the onset of diabetes, the differences became significant only after 6 weeks of diabetes.

Decreased nerve conduction velocity is a physiologic parameter indicative of nerve dysfunction consistent with the development of DPN (25). A gradual slowing of MNCV was evident after 2 weeks of diabetes in wild-type mice and was significantly different from control at most subsequent weeks (Fig. 1). Although the diabetic Cav1 knockout mice showed a significant MNCV slowing after 1 week, the temporal decline in MNCV was comparable between genotypes. However, the magnitude of the decrease in MNCV was significantly greater in the diabetic Cav1 knockout mice (20–25% decline) compared with their wild-type counterparts (12–15% decline). On the other hand, diabetes had little effect on SNCV in the wild-type



**FIG. 1.** Lack of Cav1 correlates with an increased severity in electrophysiologic and psychophysical measures of DPN. **A:** Time course of changes in MNCV and SNCV from wild-type and Cav1 knockout mice treated with vehicle or STZ. \* $P < 0.05$  compared with time-matched genotype control;  $\wedge P < 0.05$  compared with time-matched diabetic wild-type mice. Time course of changes in mechanical (**B**) or thermal (**C**) sensitivity in wild-type and Cav1 knockout mice treated with vehicle or STZ. \* $P < 0.05$  compared with time-matched genotype control;  $\wedge P < 0.05$  compared with time-matched diabetic wild-type mice. Animal numbers per treatment and genotype are listed in Table 1. KO, knockout; WT, wild type.

mice but showed a significant slowing after 12 weeks of diabetes in the Cav1 knockout mice. Importantly, the enhanced MNCV deficit in the Cav1 knockout mice was not because of a developmental neuropathy because no difference existed in MNCV between nondiabetic wild-type and Cav1 knockout mice.

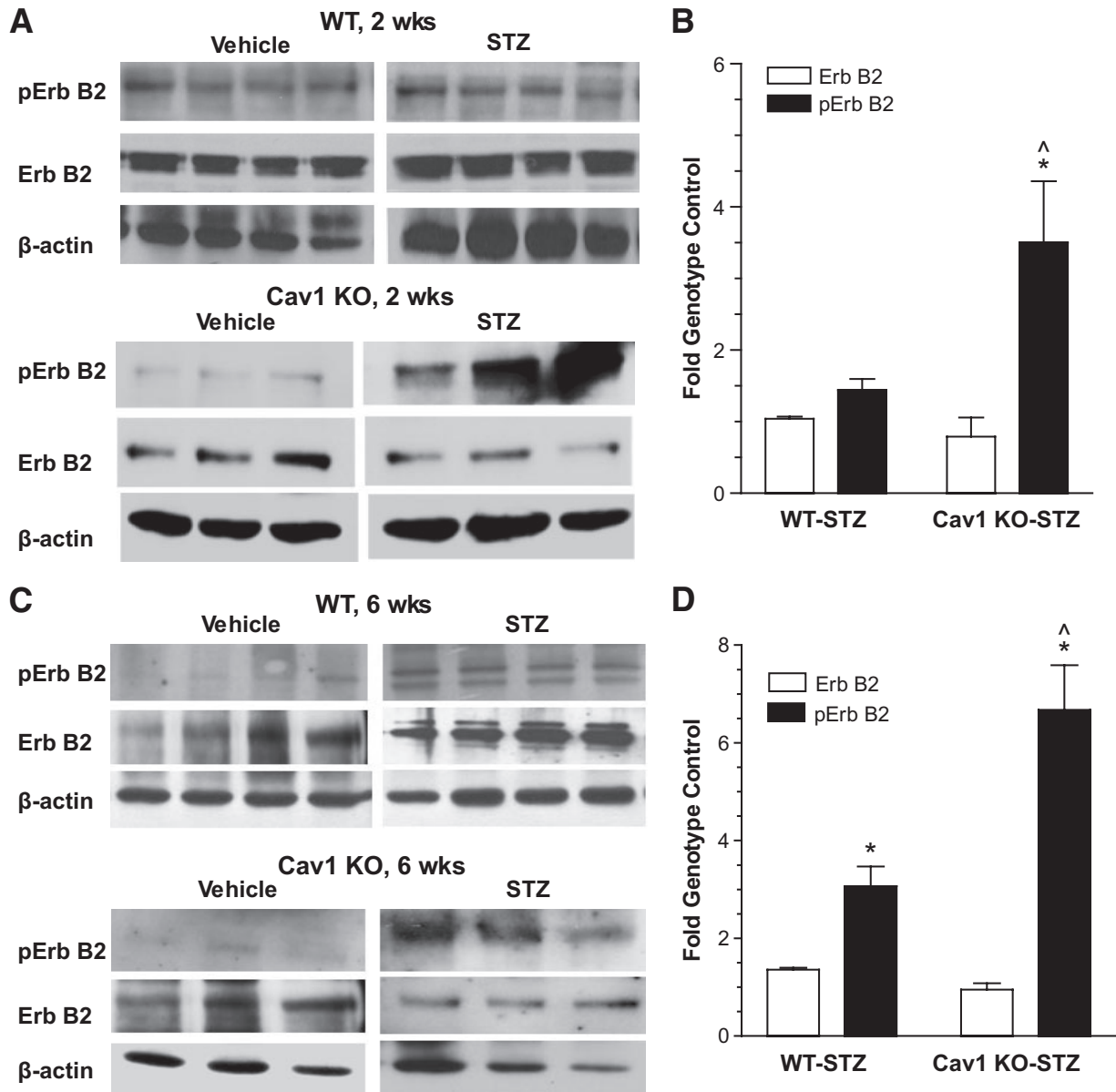
Consistent with the decrease in MNCV, diabetic wild-type mice showed a gradual onset of a mechanical hypoalgesia that was maximal within 6 weeks of diabetes (Fig. 1B). The temporal onset of the mechanical hypoalgesia in the Cav1 knockout mice was more rapid and significantly different from the diabetic wild-type mice at 1–2 weeks, but the extent of the mechanical hypoalgesia between the genotypes converged as diabetes progressed. Surprisingly, Cav1 knockout mice developed a thermal hypoalgesia that was significantly greater than that observed in the diabetic wild-type mice at most time points (Fig. 1C). This effect was not related to a greater decrease in intraepidermal nerve fiber density (iENFD) after 2 or 6 weeks of diabetes (supplemental Fig. 2).

**Erb B2 activity is increased in diabetic nerve of wild-type and Cav1 knockout mice.** After 2 weeks of diabetes, immunoblot analysis of pErb B2 indicated little change in the diabetic wild-type mice (Fig. 2A and B). In contrast, the absence of Cav1 correlated with a 3.4-fold increase in the level of pErb B2 that was not because of an increase in total Erb B2 levels. After 6 weeks of diabetes, pErb B2 levels increased about threefold in wild-type mice but remained significantly more elevated in the Cav1 knockout mice compared with its genotype control and diabetic wild-type mice (Fig. 2C and D). After 12 weeks of diabetes, pErb B2 was still more elevated in Cav1 knockout mice relative to the wild-type cohort, consistent with the conclusion that diabetes induces a prolonged activation of Erb B2 in the Cav1 knockout mice (Fig. 2E and F). Immunofluorescence analysis of sciatic nerve supported an SC localization for the increased pErb B2 immunoreactivity, since it surrounded the axonal marker neurofilament H (Fig. 2G).

**Inhibition of Erb B2 activity reverses decreased MNCV in diabetic mice.** To address whether Erb B2 activation contributed to the indexes of nerve function, mice were treated with two structurally diverse inhibitors of epidermal growth factor receptor (EGFR) family members. PKI 166 is an antagonist of EGFR family members that has been used to demonstrate the contribution of Erb B2 activation to demyelination (11,12). Similarly, erlotinib is a clinically approved inhibitor of the EGFR that also can inhibit Erb B2 receptors (26). Therefore, identical outcomes with the use of these inhibitors would support the conclusion that Erb B2 could contribute to DPN.

Wild-type and Cav1 knockout mice were rendered diabetic for 3 weeks, and subgroups were treated with 25 mg/kg of PKI 166 or vehicle biweekly for 3 weeks. This dose of PKI 166 was chosen based upon its prior efficacy in inhibiting Erb B2-mediated demyelination in mice (11). Six weeks of diabetes resulted in a decrease in MNCV in both wild-type and Cav1 knockout mice, and administering PKI 166 for the final 3 weeks improved this deficit in both diabetic genotypes (Fig. 3A). However, the magnitude of this reversal was greater in the diabetic Cav1 knockout mice given their significantly more impaired MNCV. Importantly, PKI 166 alone did not alter MNCV in either genotype, indicating that basal levels of Erb B2 activity do not affect MNCV.

Given the multiple metabolic pathways that contribute to nerve dysfunction in diabetes, it was surprising that PKI 166 was so efficacious in reversing the MNCV deficit after 6 weeks of diabetes. This outcome suggests that altered Erb B2 activity may be an important contributor to early metabolic changes that decrease MNCV. Therefore, we next determined if inhibiting Erb B2 with erlotinib was as



**FIG. 2.** Diabetes increases pErb B2 in sciatic nerve. *A, C, and E:* Upper panels show representative immunoblot analysis demonstrating induction of pErb B2 in diabetic mice after 2, 6, and 12 weeks of diabetes, respectively. *B, D, and E:* Immunoblot quantitation of Erb B2 and pErb B2 after 2, 6, and 12 weeks of diabetes, respectively (nondiabetic wild-type and Cav1 knockout [ $n = 3-4$ ]; diabetic wild-type and Cav1 knockout [ $n = 4-6$ ]). Levels of Erb B2 and pErb B2 were normalized to  $\beta$ -actin and expressed as a percent of the genotype control. \* $P < 0.05$  compared with genotype control. ^ $P < 0.05$  compared with diabetic wild-type mice. *G:* Immunofluorescence analysis of pErb B2 induction in cross section of sciatic nerve of 6-week-diabetic wild-type mouse. NF-H, neurofilament heavy chain. KO, knockout; WT, wild type. (A high-quality color digital representation of this figure is available in the online issue.)

effective in reversing MNCV deficits and sensory dysfunction in longer-term diabetic mice. Wild-type and Cav1 knockout mice were rendered diabetic, and at 12 weeks postinduction of diabetes, subgroups of the diabetic mice were treated biweekly with vehicle or 25 mg/kg erlotinib for 3 weeks. Cav1 knockout mice again developed a substantial mechanical (Fig. 3*B*) and thermal hypoalgesia (Fig. 3*C*) that was accompanied by a 20% decrease in MNCV (Fig. 3*D*). After 15 weeks of diabetes, Cav1 knockout mice that received the drug vehicle still showed substantial sensory deficits. However, erlotinib partially reversed the decrease in MNCV and improved the mechanical hypoalgesia without affecting thermal sensitivity. Although wild-type mice showed only modest activation of Erb B2 after 12 weeks of diabetes, erlotinib partially

corrected the MNCV deficit (Fig. 3*E*) and had an identical effect on improving the mechanical but not thermal hypoalgesia (data not shown). Together, the above data suggest that activation of Erb B2 contributes to the decline of myelinated fiber function in DPN.

**Erb B2 activation is sufficient to mimic aspects of diabetic neuropathy.** Although PKI 166 and erlotinib attenuated some of the sensory deficits, they may inhibit other EGFR family members and do not specifically target Erb B2 receptors localized to SCs. Because diabetes alters many aspects of nerve physiology, we conditionally expressed a constitutively active Erb B2 (caErb B2) in myelinated SCs to determine if Erb B2 was sufficient to contribute to a sensory neuropathy in the absence of diabetes.

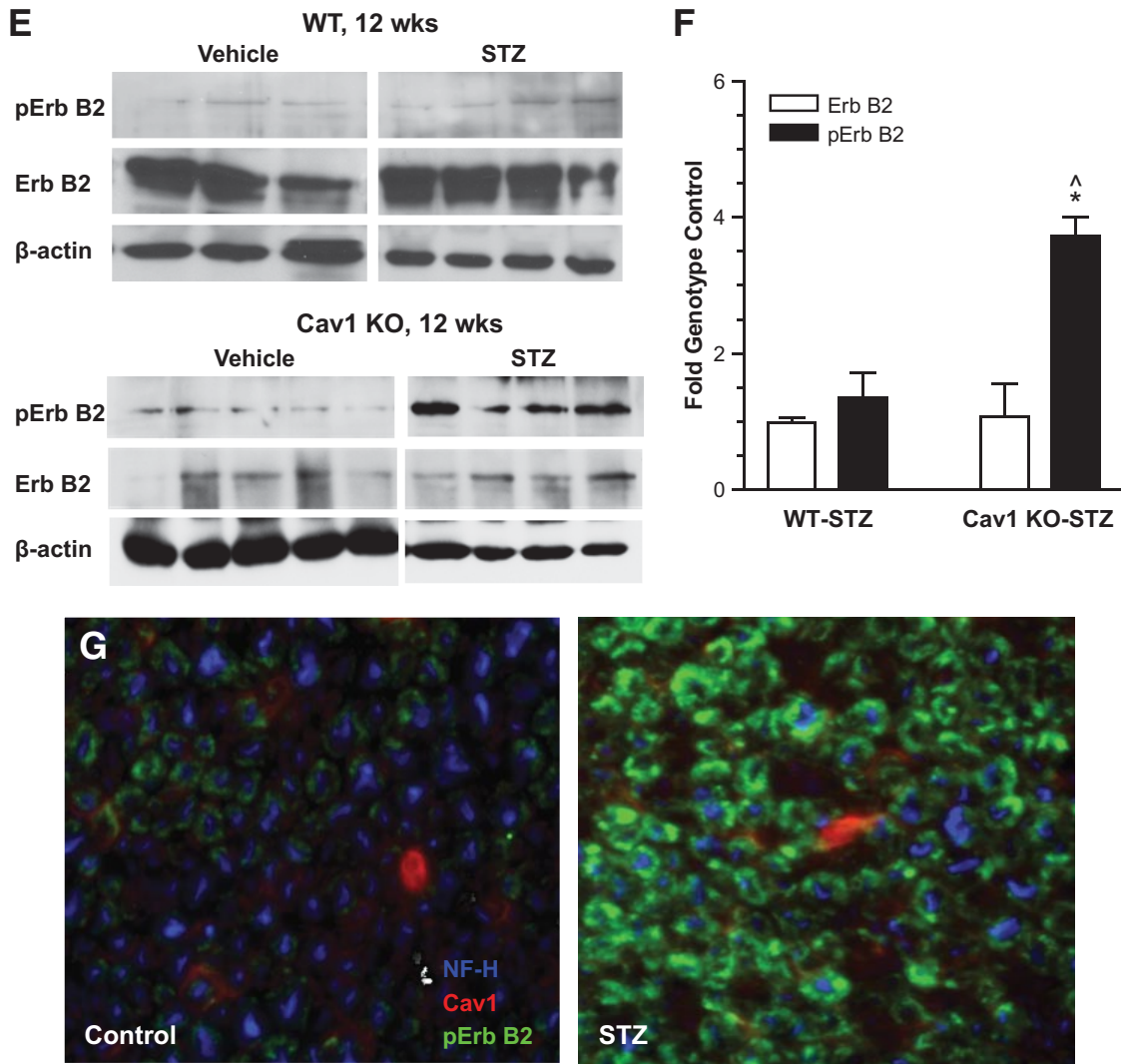


FIG. 2. Continued.

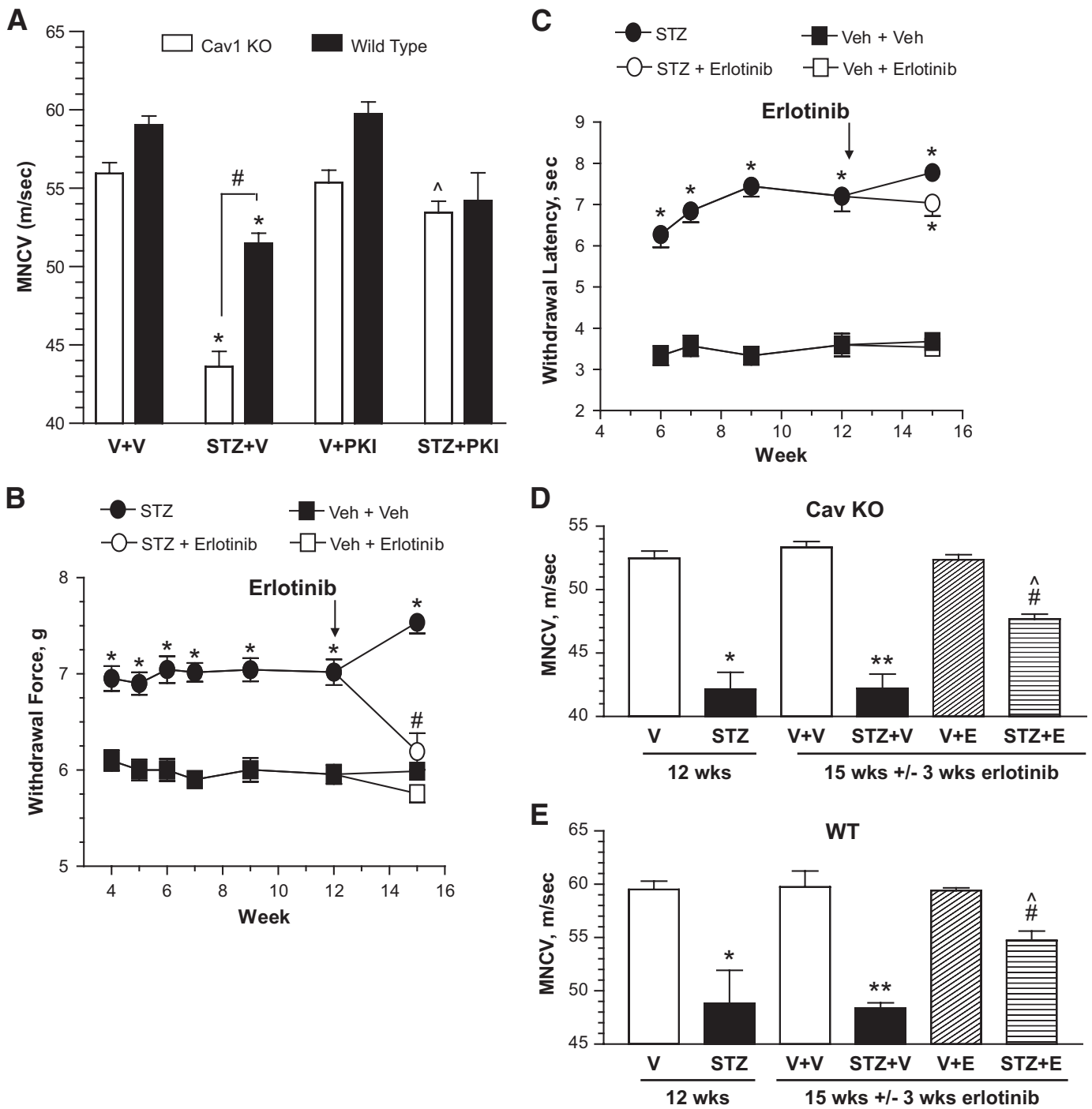
The conditional transgenics were generated by placing the rtTA transgene under the control of the rat  $P_0$  promoter (22). Enrichment of rtTA transcripts in sciatic nerve was verified by reverse transcriptase PCR analysis of RNA isolated from various tissues of the progeny from one founder line (Fig. 4A). This mouse was bred with a second transgenic line that broadly expresses the caErb B2 (21), and PCR analysis of genomic DNA was used to identify bitransgenic progeny (Fig. 4B and C). Mice hemizygous for both transgenes showed an increase in pErb B2 and total Erb B2 in sciatic nerve after addition of doxycycline (DOX) to the diet (Fig. 5A). Transgene induction was readily reversed upon removal of the DOX diet (Fig. 5B), and no gross phenotypic differences or changes in weight gain were observed between the groups receiving standard rat chow or the DOX diet (supplemental Fig. 3A).

Induction of the caErb B2 by the DOX diet led to the development of a significant mechanical hypoalgesia (Fig. 6A) and decrease in MNCV (Fig. 6B). Compared to either the baseline response measured at week 0 or the time-matched controls maintained on standard rat chow, the severity of the mechanical hypoalgesia progressively increased over 9 weeks but was reversed by removing the DOX diet. Similarly, the MNCV deficit was not a nonspecific effect of DOX because wild-type mice placed on the

DOX diet did not show a change in MNCV (supplemental Fig. 3B). Indeed, the MNCV deficit was clearly related to transgene induction because it was reversed by removing the DOX diet or by treating the mice with 25 mg/kg PKI 166 (Fig. 6C). Importantly, any leaky expression of the caErb B2 was not sufficient to induce a developmental neuropathy since bitransgenic mice on standard rat chow showed a very consistent response to mechanical stimulation over the entire time course (Fig. 6A). Additionally, transgene induction had no effect on SNCV (Fig. 6B) or thermal sensitivity (supplemental Fig. 3C).

## DISCUSSION

A number of elegant pharmacologic and genetic studies have defined the necessity of the neuregulin/Erb B ligand/receptor pair in providing a complex array of signals that support survival, growth, and differentiation of SCs (27–29). However, neuregulins may also promote demyelination (30,31), and pathological activation of neuregulin/Erb B2 signaling may be physiologically relevant in the generation of sensory neuropathies independent of the diabetic phenotype (13,32). Our study extends these observations and provides the initial identification that activation of Erb

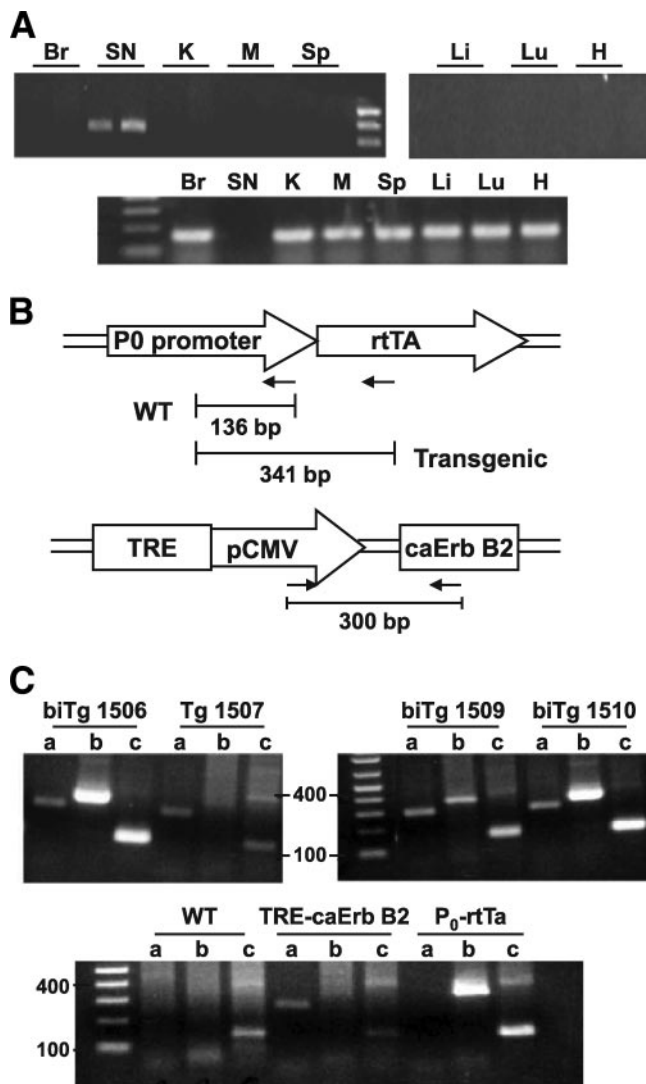


**FIG. 3.** Inhibition of Erb B2 with PKI 166 or erlotinib attenuates specific indexes of diabetic sensory neuropathy. **A:** Wild-type and Cav1 knockout mice were rendered diabetic for 3 weeks then treated with 25 mg/kg PKI 166 or the drug vehicle biweekly for 3 weeks. MNCV was assessed after 6 weeks as described in the text ( $n = 5-7$  per group per genotype). \* $P < 0.01$  compared with genotype control; # $P < 0.001$  compared with diabetic wild-type mice; <sup>^</sup> $P < 0.001$  compared with diabetic Cav1 knockout. **B-E:** Wild-type and Cav1 knockout animals were rendered diabetic for 12 weeks then treated with 25 mg/kg of erlotinib ( $n = 7$ ) or the drug vehicle ( $n = 7$ ) biweekly for 3 weeks. Erlotinib reversed the mechanical (**B**) but not the thermal (**C**) hypoalgesia observed after 15 weeks of diabetes in the Cav1 knockout mice. \* $P < 0.001$  compared with control; # $P < 0.05$  compared with 12-week diabetic. Cav1 knockout (**D**) and wild-type (**E**) ( $n = 14$ ) were rendered diabetic for 12 weeks, and MNCV was assessed ( $n = 4$ ) in each genotype. Subgroups ( $n = 5$ ) received vehicle (V) or erlotinib (E) biweekly for 3 weeks, and MNCV was assessed. \* $P < 0.001$  compared with vehicle. \*\* $P < 0.001$  compared with V+V control. # $P < 0.05$  compared with STZ+V. <sup>^</sup> $P < 0.05$  compared with V+E. KO, knockout; WT, wild type.

B2 in diabetic nerve contributes to the progression of DPN.

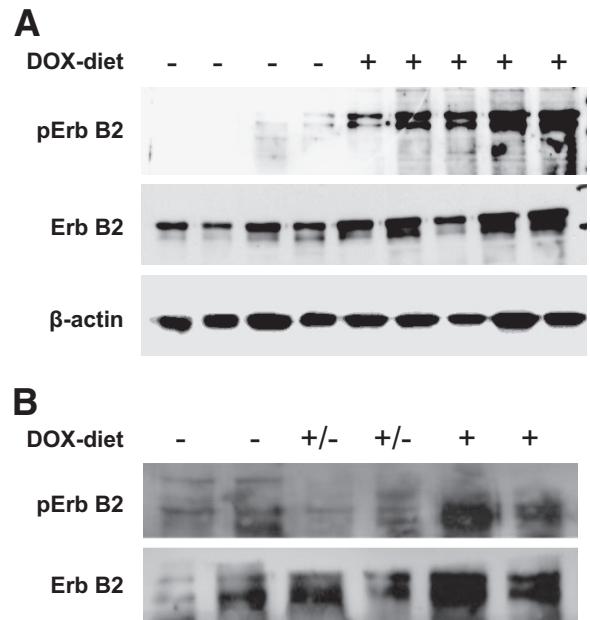
**Erb B2 and the diabetic nerve.** Erb B2 activation correlated with a decrease in MNCV and the development of a mechanical and thermal hypoalgesia in diabetic wild-type and Cav1 knockout mice. Because pharmacolog-

ical inhibition of Erb B2 reversed the MNCV deficit and mechanical hypoalgesia, Erb B2 activation contributes primarily to these particular indexes of DPN. This reversal is unlikely to be because of inhibition of either the EGFR or Erb B4 as previous studies have shown these receptors are poorly expressed or absent in adult SCs (33,34).



**FIG. 4.** Verification of the tissue specificity of the rtTA transgene and generation of P<sub>0</sub>-rtTA × caErb B2 bitransgenics. **A:** mRNA was isolated from various tissues obtained from a progeny of one founder line, and cDNA was prepared. The rtTA transcript was amplified by PCR using 2 and 4 μg of total cDNA. The β-actin transcript was amplified by PCR using 2 μg of total cDNA. The rtTA transcript was enriched in samples from sciatic nerve despite a low level of β-actin. Br, brain; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; SN, sciatic nerve; Sp, spleen. **B:** Schematic of PCR strategy for identifying P<sub>0</sub>-rtTa and TRE-caErb B2 transgenes. **C:** Identification of several bitransgenic (biTg) and one nonbiTg progeny from cross of P<sub>0</sub>-rtTa and TRE-caErb B2 parents. Bottom panel shows the presence of transgenes in the parents but their absence in a wild-type (WT) mouse. Amplicons identify the TRE-caErb B2 (lane a) and P<sub>0</sub>-rtTA (lane b) transgenes. Lane c is positive control for the presence of the endogenous P<sub>0</sub> promoter.

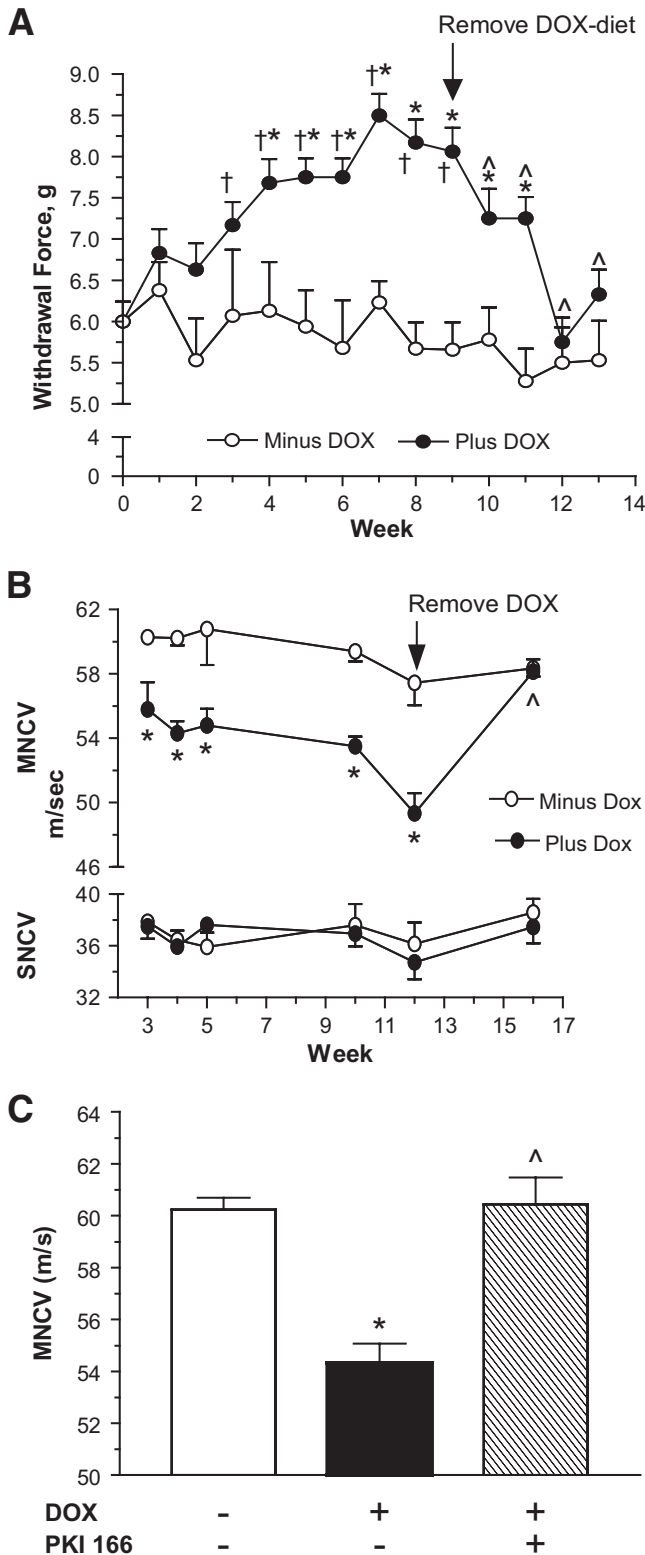
However, Erb B2 has been reported in lumbar DRG neurons (35), and we cannot rule out that inhibition of this population of receptors contributes to changes in MNCV in diabetes. Nonetheless, activation of Erb B2 in SCs is at least sufficient to induce a mechanical hypoalgesia and decrease MNCV because induction of a constitutively active Erb B2 in myelinated SCs recapitulated this aspect of DPN while sparing any effects on SNCV and sensitivity to thermal stimulation of the planta pedis. Because detection of mechanical stimuli is mediated primarily by myelinated axons associated with Aδ fibers (36,37), the above results are consistent with the enhanced expression of the caErb B2 in myelinated axons. Although Aδ fibers may mediate sensitivity to thermal stimuli, these fibers primar-



**FIG. 5.** Induction of caErb B2 in sciatic nerve of bitransgenic mice. **A:** Bitransgenic mice were placed on standard rat chow (-) or the DOX diet (+) for 9 weeks. Sciatic nerves were harvested, and immunoblot analysis was performed for pErb B2, Erb B2, and β-actin. **B:** Bitransgenic mice were placed on standard rat chow (-) or the DOX diet (+) for 9 weeks, and the DOX diet was replaced (±) with standard rat chow for 3 weeks. Sciatic nerves were harvested, and immunoblot analysis was performed for pErb B2 and Erb B2.

ily contribute to foot withdrawal in response to a higher heating rate than was used in our study (38). Additionally, it is also noteworthy that early treatment with PKI 166 totally reversed the MNCV deficit observed at 6 weeks in both diabetic wild-type and Cav1 knockout mice. Given the multiple pathways that have been implicated in contributing to the development of MNCV deficits in diabetic nerve, it is surprising that inhibiting Erb B2 would be capable of such a prophylactic effect. Although we observed a significant change in mechanical sensitivity at 6 weeks, this early nerve conduction deficit may be related to acute glucotoxicity rather than bonafide DPN (39). More in line with our expectation, after 15 weeks of diabetes, addition of erlotinib for the final 3 weeks only partially reversed the MNCV deficit and improved mechanical sensitivity, suggesting contributions from other pathways. Although erlotinib did not improve the thermal hypoalgesia, we cannot exclude that the drug may alter SNCV and iENFD because these parameters were not substantially altered in the diabetic mice.

An intriguing aspect of our findings relates to the role of the pathologic activation of Erb B2 in promoting demyelination (11,12,32). Although myelin thinning can be observed in sural nerve of long-term (9 months) diabetic mice (23), we observed no major changes in expression of P<sub>0</sub> in sciatic nerve at the earlier time points used in our study (supplemental Fig. 4). The contribution of Erb B2 activation to rapid demyelination after axotomy (11) or infection with leprosy bacilli (12) may result from preferential activation of the p42/p44 mitogen-activated protein kinase (MAPK) that has been implicated in promoting demyelination (12,31). Although activation of p42/p44 MAPKs has been observed in DRG from 8- to 12-week diabetic rats (40,41), p42/p44 MAPKs did not increase in activity in sural nerve (41). It is possible that rapid



**FIG. 6.** Induction of caErb B2 induces a mechanical hypoalgesia and decreased MNCV. **A:** Bitransgenic mice ( $n = 12$ ) were assessed for baseline mechanical sensitivity at week 0. Animals were placed on standard rat chow ( $n = 5$ ) or the DOX diet ( $n = 7$ ), and mechanical sensitivity was measured weekly.  $*P < 0.05$  compared with time-matched control;  $\dagger P < 0.05$  compared with week 0. The mechanical hypoalgesia improved upon withdrawal of the DOX diet after 9 weeks (arrow).  $^{\wedge}P < 0.01$  compared with response at 9 weeks on DOX diet. **B:** Bitransgenic mice were maintained on the DOX diet ( $n = 5-7$ ) or standard rat chow ( $n = 5-7$ ) for the indicated time, and MNCV and SNCV were assessed. After 12 weeks, the DOX diet was replaced with

activation of Erb B2, such as occurs after axotomy (11), may preferentially couple to pathways promoting demyelination or that reparative mechanisms do not have sufficient time to counteract/attenuate the degenerative signals. Because our evidence clearly supports that Erb B2 activation is sufficient to contribute to the neurophysiological deficits associated with a more chronic and prolonged stress induced by diabetes, less robust degenerative signals may be generated and/or the dynamic repair of nerve dysfunction in early stage DPN may help avoid overt demyelination. However, longer-term pharmacologic studies are needed to determine if Erb B2 may contribute to myelin thinning and decreased axonal caliber. Although the mechanism by which Erb B2 may alter MNCV is unclear, recent data suggests that Erb B2 can increase p38 MAPK activity and upregulate matrix metalloproteinase nine (42). Because inhibition of p38 MAPK improves nerve conduction velocity deficits (43), it is tempting to speculate that Erb B2 may provide an upstream signal that contributes to p38 MAPK activation.

**A role for Cav1 in regulating SC signaling by neuregulins.** Cav1 is upregulated during myelination (44), but its role in SC function remains poorly defined. Although Cav1 may help organize components of the myelin membrane because of its ability to serve as a scaffolding protein (45), this function is not necessary for myelination because peripheral nerves from adult Cav1 knockouts show little morphologic difference compared with wild-type nerve. However, Cav1 is also known to regulate cell signaling cascades in glial cells (45), and its downregulation enhanced neuregulin-induced demyelination of SC/DRG neuron cocultures (18). Although Cav1 expression is downregulated in both diabetic kidney (46) and nerve (17), it remained unclear whether it may affect the physiological progression of DPN and if this may be related to altered Erb B2 activity. Our data suggest that Cav1 does play a modulatory role in the development of specific aspects of DPN because the Cav1 knockout mice developed a more severe deficit in MNCV that correlated with an increase in Erb B2 activation. Erb B2 activation contributed to both the MNCV deficits and mechanical hypoalgesia because they were reversed by PKI 166 or erlotinib. Although these data suggest that Cav1 may serve as an endogenous regulator of the pathologic activation of Erb B2, its expression does not affect the basal activity of Erb B2 because no differences were noted in MNCV or mechanical sensitivity between nondiabetic knockout and wild-type mice. This discrepancy raises the possibility that changes in Erb B2 localization may be necessary for producing degenerative signals and that the absence of Cav1 in this compartment may directly or indirectly affect receptor activation.

It is surprising that the diabetic Cav1 knockout mice showed an enhanced magnitude of thermal hypoalgesia compared with the diabetic wild-type animals. Altered Erb B2 activity could not account for this difference because erlotinib was unable to promote recovery of the thermal hypoalgesia. Similarly, Erb B2 induction did not alter thermal sensitivity in the bitransgenic mice. At the heating rate and maximum temperature threshold ( $\sim 42^{\circ}\text{C}$ ) used in

standard rat chow for 3 weeks ( $n = 5$ ).  $*P < 0.05$  compared with time-matched control minus DOX diet;  $^{\wedge}P < 0.01$  compared with plus DOX at 12 weeks. **C:** Bitransgenic mice were given standard rat chow ( $n = 7$ ) or the DOX diet for 4 weeks. DOX-treated animals received either vehicle ( $n = 5$ ) or 25 mg/kg PKI 166 ( $n = 5$ ) biweekly for 3 weeks, and MNCV was measured.  $*P < 0.05$  compared with minus DOX diet.  $^{\wedge}P < 0.01$  compared with plus DOX and drug vehicle.



our study, unmyelinated C fibers should primarily be activated (36). Because thinly myelinated type II A $\delta$  fibers can have some overlapping thermal sensitivity with C fibers (36), it is possible that loss of Cav1 may affect thermal nociceptors in these afferents. However, to the best of our knowledge, Cav1 is not expressed in the terminals of small- to medium-diameter sensory neurons and is not known to affect vanilloid receptors. Although loss of iENFD can contribute to thermal hypoalgesia, no fiber loss was observed in the 6-week-diabetic wild-type or Cav1 knockout mice. Thus, the enhanced thermal hypoalgesia is possibly related to metabolic differences between the genotypes.

In summary, considerable evidence supports that an altered neurotrophism contributes to sensory neuron degeneration in DPN (9). We provide genetic and pharmacological evidence that pathological activation of Erb B2 receptors in SCs also contributes to the pathophysiological progression of DPN. Given the critical role of neuregulin in SC biology, we propose that an altered neuregulinism may contribute to axo-glia dysfunction and affect responses mediated by small, myelinated afferents. Further, as diminished tactile sensitivity is a feature of DPN, it will be important to determine if changes in Erb B2 signaling may also impact larger A $\beta$  fibers innervating Meissner or Pacinian corpuscles (47). Targeting Erb B2 signaling may provide a novel therapeutic approach toward ameliorating some of the symptoms associated with DPN in humans.

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