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TRPV1$^+$ Sensory Neurons Control β Cell Stress and Islet Inflammation in Autoimmune Diabetes

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SUMMARY

In type 1 diabetes, T cell-mediated death of pancreatic β cells produces insulin deficiency. However, what attracts or restricts broadly autoreactive lymphocyte pools to the pancreas remains unclear. We report that TRPV1$^+$ pancreatic sensory neurons control islet inflammation and insulin resistance. Eliminating these neurons in diabetes-prone NOD mice prevents insulitis and diabetes, despite systemic persistence of pathogenic T cell pools. Insulin resistance and β cell stress of prediabetic NOD mice are prevented when TRPV1$^+$ neurons are eliminated. TRPV1^{NOD}, localized to the *Idd4.1* diabetes-risk locus, is a hypofunctional mutant, mediating depressed neurogenic inflammation. Delivering the neuropeptide substance P by intra-arterial injection into the NOD pancreas reverses abnormal insulin resistance, insulitis, and diabetes for weeks. Concordantly, insulin sensitivity is enhanced in *trpv1*$^{-/-}$ mice, whereas insulitis/diabetes-resistant NODxB6*Idd4*-congenic mice, carrying wild-type TRPV1, show restored TRPV1 function and insulin sensitivity. Our data uncover a fundamental role for insulin-responsive TRPV1$^+$ sensory neurons in β cell function and diabetes pathoetiology.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease governed by multiple genetic and environmental risk factors. Overt diabetes reflects glucose intolerance due to insulin deficiency. It is the end result of prediabetes, with progressive lymphoid infiltration around and then inside pancreatic islets of Langerhans and subsequent destruction of insulin-producing β-cells by autoreactive T lymphocytes (Anderson and Bluestone, 2005). T1D is characterized by a permissive immune system that fails to impose tolerance to arrays of self-antigens. Although the initiating events are not fully understood, β cell stress and death in the course of early islet restructuring are thought to provide sensitizing autoantigens, which expand autoreactive T cell pools in pancreatic lymph nodes (Mathis et al., 2001; Rosmalen et al., 2002; Trudeau et al., 2000; Zhang et al., 2002).

Self-antigens targeted in T1D are expressed in β cells and, in most cases, elsewhere in the body. They prominently include neuronal antigens, recognized by T cells with pathogenic potential (Salomon et al., 2001; Winer et al., 2001). It is unclear why, in T1D, T cells infiltrate only islets and their associated glia (Winer et al., 2003). It is also unclear whether autoimmunity and islet inflammation are related to hyperinsulinism and insulin resistance typical for even young NOD mice (Amrani et al., 1998; Chaparro et al., 2006).

There is evidence for functional interactions between nervous and immune systems (e.g., Wang et al. [2003]), but connections between islet autoimmunity and the nervous system remain ill defined (Carrillo et al., 2005). The interface between nervous system and external and tissue environments is the primary sensory afferent neuron. Primary afferents also have efferent function through local release of mediators such as neuropeptides (e.g., substance P [sP], CGRP). Islets may be innervated by primary sensory neurons, but their local function is uncertain (Ahren, 2000). A prominent subset of sensory neurons expresses the transient receptor potential vanilloid-1 (TRPV1) protein, a nonspecific cation channel that was

first identified as the receptor for capsaicin (Caterina et al., 2000; Prescott and Julius, 2003). TRPV1$^+$ neurons are of known importance in proinflammatory reactions (O'Connor et al., 2004), islet infiltrating lymphocytes express receptors for neuropeptides (Persson-Sjogren et al., 2005), and we asked if these sensory neurons may have a role in T1D.

RESULTS

TRPV1$^+$ Sensory Afferents Control Onset of Islet Inflammation and Diabetes

Using immunofluorescence, we determined that murine islets are associated with meshworks of TRPV1$^+$ fibers (Figure 1A). TRPV1 was undetectable in endocrine islet cells by immunofluorescence (Figures 1A and 1B) and by RT-PCR of purified NOD islets (Figure S1 in the Supplemental Data available with this article online). Based on this evidence of islet innervation by TRPV1$^+$ primary afferent sensory neurons, we investigated their possible role in T1D pathogenesis, using neonatal treatment of diabetes-prone NOD mice with capsaicin to permanently remove these neurons (Caterina and Julius, 2001; Jancso et al., 1977). Two-day-old NOD mice received capsaicin (50 mg/kg, s.c.) or vehicle (NOD^{caps}, NOD^{ctrl}). As expected from the voluminous literature, capsaicin-treated mice were viable, fertile, and without abnormalities in growth or gross tissue structure, including pancreas. We confirmed the lack of TRPV1 expression in NOD^{caps} mice, using immunofluorescence (Figures 1A and 1B, TRPV1 green), western blots (8, 12, and 20 week), and standard hot-plate testing in randomly selected 5- to 6-week-old mice or in diabetic animals (Figure 1C). Consistent with loss of neuropeptide secreting TRPV1$^+$ neurons, NOD^{caps} mice showed no sP staining (Figure S2).

Islet infiltration by hematopoietic inflammatory cells begins by 4–5 weeks of age, accumulating at the peri-islet Schwann cell (pSC) border; the autoimmune destruction of the pSC mantle is extensive by 8 weeks of age, 8–12 weeks before the onset of overt diabetes (Winer et al., 2003). In NOD^{caps} mice, islet infiltrations were significantly reduced, compared with NOD^{ctrl} (Figure 1D, $p < 0.0001$). More than 70% of NOD^{caps} islets were free of lymphocytes by the age of 20 weeks, whereas even in nondiabetic NOD^{ctrl} mice of that age, very few islets were lymphocyte free (Figure 1D). In one-third of NOD^{caps} mice, infiltrated islets were entirely absent, as demonstrated by extensive, serial sectioning. In the remaining two-thirds of NOD^{caps} mice, most islets remained free of lymphocytes, with some degree of inflammation in rare islets, but strikingly, there was little of the typical insulitis progression over time (Figure 1E). Concordantly, capsaicin treatment delayed the onset of diabetes ($p = 0.0002$) and reduced its incidence (Figure 1F, $p < 0.0001$, 35 weeks, $n = 52$ mice/group, life table analysis). No further NOD^{caps} mice developed disease over the next 12 weeks, with about 80% reduction in final diabetes incidence ($p < 0.0001$, Fisher's exact test).

NOD mice spontaneously develop a Sjögren-like sialitis/lacrimitis, which is under genetic controls separate from diabetes (Boulard et al., 2002; Cha et al., 2002). NOD^{caps} mice exhibited the same submandibular lymphocyte infiltrates as NOD^{ctrl} (Figure S3). Capsaicin treatment thus causes a dramatic reduction in pancreatic islet inflammation and development of diabetes without a generalized effect on autoimmune infiltrations elsewhere in the NOD mouse.

Capsaicin treatment effects on islet infiltration and disease development could reflect a failure to generate islet autoreactive T cell pools, a block of their tissue accumulation, or changes in immunoregulatory mechanisms. Capsaicin was reported to affect some immune functions in other animal models (Chancellor-Freeland et al., 1995; Helme et al., 1987; Nilsson et al., 1991; Santoni et al., 1996), and we compared development and functions of systemic (Figures 2A–2E) and pancreatic T cells (Figures 2F–2I) in NOD^{caps} and NOD^{ctrl} mice. Systemic T cell pools autoreactive to islet- (Insulin, GAD65), pSC- (GFAP, S100β), and other disease-associated antigens (HSP60, BSA) (Winer et al., 2003) were indistinguishable in NOD^{caps} and NOD^{ctrl} spleen cells (Figure 2A). To probe the development of diabetogenic T cell pools in NOD^{caps} mice, we measured the peripheral frequency of pathogenic CD8$^+$ T cells that recognize residues 206–214 of the glucose-6-phosphatase catalytic subunit related protein (IGRP) and its homologous, high-avidity mimotope, NRP-V7 (Amrani et al., 2000, 2001; Anderson et al., 1999; Lieberman et al., 2003; Verdaguer et al., 1996, 1997). The size of the circulating NRP-V7-reactive CD8$^+$ T cell pool was similar in NOD^{caps} and NOD^{ctrl} spleens ($0.25\% \pm 0.1\%$, $p = 0.69$) (Figure 2D). Lymphoid organ cellularities and T cell subset distributions were also not different in NOD^{caps} and NOD^{ctrl}, comparing splenocytes, axillary lymph nodes, and thymus (Figure S4). Delayed-type hypersensitivity reactions developed normally in NOD^{caps} mice (Figure S5), suggesting intact antigen presentation and effector cell generation (Cua et al., 1995; Morikawa et al., 1993).

In contrast, pancreatic NOD^{caps} lymph node tissue contained significantly reduced proportions and absolute numbers of CD8$^+$ and of activated CD8$^+$CD69$^+$ effector T lymphocytes critical for islet destruction (DiLorenzo et al., 1998) (Figures 2F and 2G). As a hallmark of prediabetes progression, prediabetic NOD mice selectively lose CD4$^+$CD25$^+$ and Foxp3$^+$ regulatory T cell subsets in pancreatic lymph node tissue (Bluestone and Tang, 2005; Pop et al., 2005). However, NOD^{caps} mice maintained their regulatory T cell compartment in pancreatic lymph nodes beyond 12–16 weeks of age (Figures 2H and 2I). Thus, there are significant differences in the pancreatic, local immune system of NOD^{caps} and NOD^{ctrl} mice, consistent with the absence of chronic progressive islet inflammation in these animals.

Conceivably, undetected abnormalities in the NOD^{caps} immune system might have influenced T cell pathogenicity. NOD^{caps} animals that did develop disease had severe

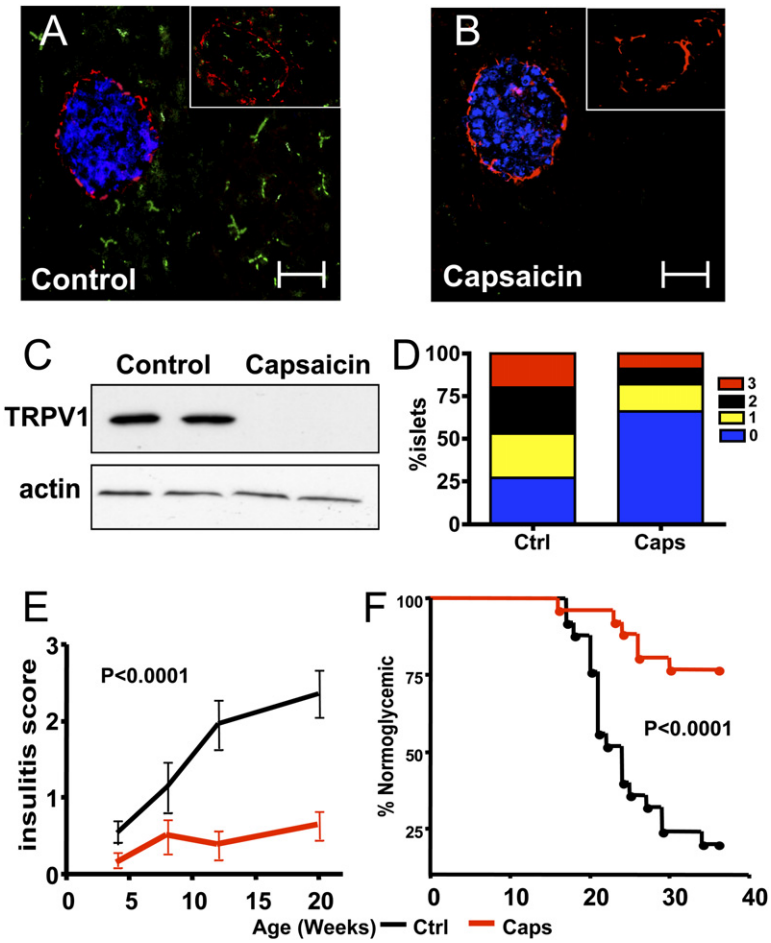

Figure 1. Removal of TRPV1$^+$ Neurons Reduces Islet Infiltration and Diabetes Progression

Immunohistochemistry of TRPV1$^+$ neurons in pancreas of 8-week-old NOD^{ctrl} (A) and NOD^{caps} (B). Insulin, blue; GFAP, red; and TRPV1, green; insert, dual-color stain for TRPV1 and GFAP in an adjacent, serial section (A). Western blot analysis of TRPV1 expression in spinal cord protein extracts from NOD^{ctrl} and NOD^{caps} mice at 12 (first lane) and 20 weeks of age (second lane) (C). Insulitis was scored in at least 300 islets from NOD^{ctrl} and from NOD^{caps} mice, 20 weeks of age (n = 5/group) (D). Kinetics of insulitis (E) and diabetes development in NOD^{ctrl} and NOD^{caps} mice (F).

insulitis, and spleen cells from these animals transferred T1D with normal kinetics to lymphocyte-free NOD.*scid* recipients not treated with capsaicin (Figure S6). We also compared the ability of splenocytes from randomly selected NOD^{caps} and NOD^{ctrl} to initiate insulitis in such NOD.*scid* mice. NOD^{caps} and NOD^{ctrl} splenocytes initiated insulitis equally (Figure 2C). Moreover, we analyzed BDC2.5 T cell receptor transgenic NOD mice treated with capsaicin (BDC2.5^{caps}) (Ji et al., 1999). BDC2.5^{caps} splenocytes transferred T1D to NOD^{ctrl} mice ($p > 0.1$). Low-dose cyclophosphamide accelerates NOD diabetes by multiple mechanisms (Hadaya et al., 2005). Low-dose cyclophosphamide accelerated diabetes development in both NOD^{caps} and NOD^{ctrl} (p = ns, Figure 2E) and was associated with reversal of the regulatory T cell accumulation present previously in NOD^{caps} versus NOD^{ctrl} pancreatic lymph nodes (Figure S7). Thus, NOD^{caps} mice retain the principal ability to generate diabetogenic T cell pools.

Collectively, our observations separate loss of self-tolerance from target tissue invasion as distinct elements of T1D pathogenesis, and they demonstrate that the NOD^{caps} immune system retains pathogenic potential. TRPV1$^+$ sensory neurons thus appear critical for the immune-cell accumulation in the pancreas.

NOD *trpv1* Is Polymorphic

The above findings identify an important role of TRPV1$^+$ primary afferent neurons in the initiation and progression of islet inflammation. TRPV1 maps to the *Idd4.1* NOD diabetes-risk sublocus (mouse chromosome 11) into an ~0.3 cM interval downstream of D11Ndsl (Figure 3A) (Grattan et al., 2002; Ivakine et al., 2005; McAleer et al., 1995). Congenic replacement of the NOD *Idd4* locus with the homologous B6 genomic interval protects from insulitis and, consequently, diabetes, although splenocytes from these congenic animals transfer both insulitis and diabetes to NOD.*scid* mice (Grattan et al., 2002). The NOD *Idd4* risk locus differs from the homologous genomic region in the insulitis- and diabetes-resistant NOR strain, which carries nearly 90% of the NOD genome, including most other T1D risk loci (Ivakine et al., 2005; Serreze et al., 1994).

We cloned and sequenced TRPV1 cDNA from NOD and NOR mouse dorsal root ganglia (DRG) and confirmed selected sequence regions in NOD and NOR genomic DNA. The NOR *trpv1* was identical to the published wild-type (B6, DBA) sequence, but the NOD sequence has two in-frame base exchanges, leading to predicted $P_{322} \rightarrow A_{322}$ and $D_{734} \rightarrow E_{734}$ amino acid replacements

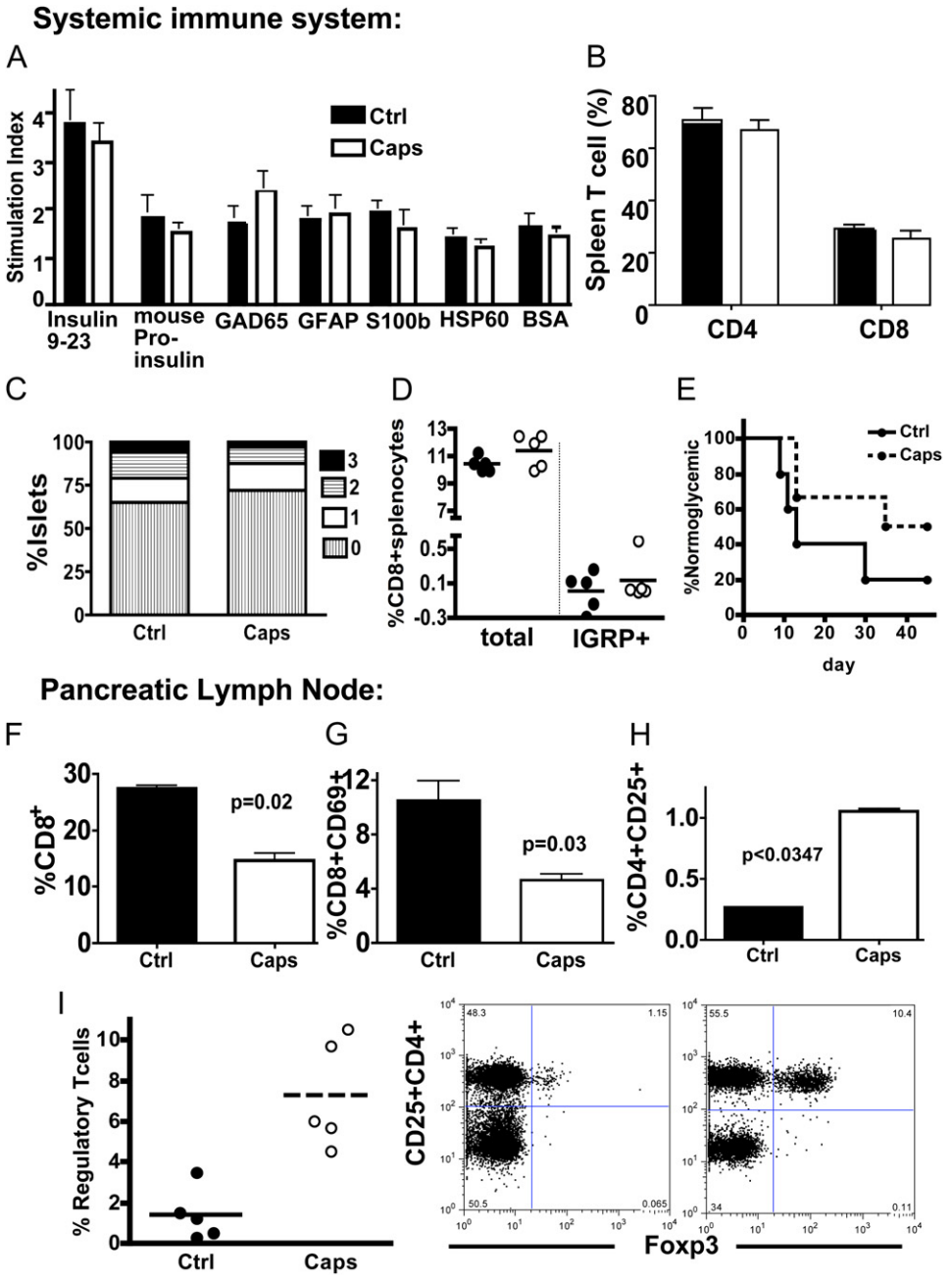

Figure 2. T Cells in NOD^{caps} and NOD^{ctrl}

Splenic T cell proliferative responses to T1D-associated antigens; comparable results were obtained at different ages (A). Pooled flow cytometry data of CD4$^+$ (FITC) and of CD8$^+$ (PE) splenic T cells, gated on CD3$^+$ (APC) cells (n = 9/group) (B). Adoptive transfer of insulitis to NOD.*scid* mice by 6- to 8-week-old NOD^{caps} or NOD^{ctrl} splenocytes (n = 10/group) (C). Tetramer staining of IGRP TCR-specific T cells among CD8+ splenocytes, 8 weeks of age (n = 5) (D). Incidence of cyclophosphamide-induced diabetes (n = 6/group) (E), CD8$^+$ (F), activated CD8$^+$CD69$^+$ (G), and CD4$^+$CD25$^+$ (H), and FoxP3$^+$CD25$^+$CD4$^+$ T cells in pancreatic lymph nodes (I). Error bars represent + one SD.

(Figure 3). Both replacements fall into regions highly conserved among diverse species (Figure 3).

TRPV1^{NOD} Is Dysfunctional

We investigated whether the sequence differences in TRPV1^{NOD} might cause abnormalities of TRPV1 function. The innervation of skin by TRPV1$^+$ sensory afferents allowed assessment of potential functional differences by whole-animal experiments in which these afferents were stimulated by subcutaneous capsaicin application (Figure 4). Before testing capsaicin, we found that there were no differences between NOD and NOR mice in basal

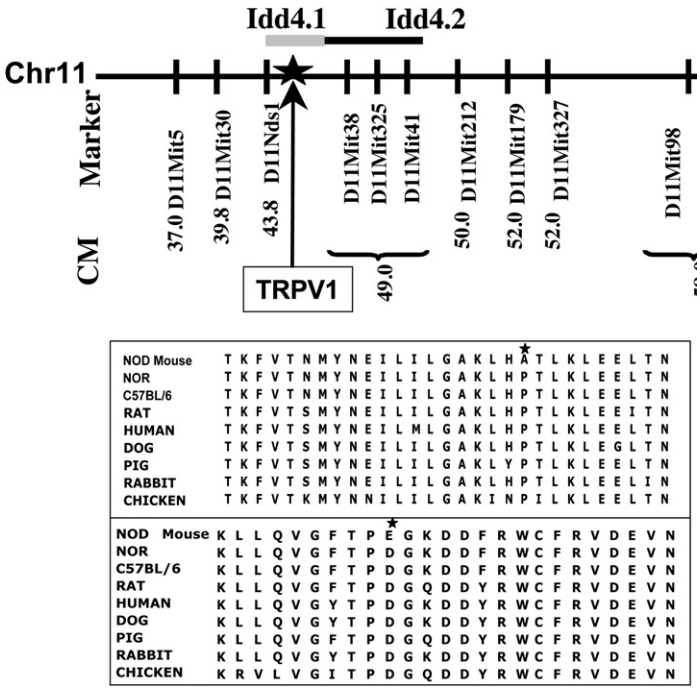

Figure 3. *trpv1* Mutations in NOD

trpv1 is located on mChr11 at 44.1 cM in the NOD *Idd4.1* locus between D11Nds1 and D11Mit38 (top). Translated protein sequence of TRPV1^{NOD} showing amino acid exchanges P322 → A322, D734 → E734, and corresponding regions in other species (bottom).

withdrawal responses to heat stimulation of the paw or tail (Figure 4A), indicating no generalized alteration of basal nociception in NOD mice. In addition, the sensitization of heat-evoked withdrawal responses after subcutaneous capsaicin administration was not different in NOD versus NOR mice (Figure 4B). However, nociceptive behavioral responses (biting, licking) evoked by subcutaneous capsaicin were markedly depressed in NOD as compared with NOR mice ($p < 0.05$, Figure 4C). Similarly, the paw edema produced by capsaicin was significantly reduced in NOD mice ($p < 0.01$, Figure 4D), implying reduced neuropeptide secretion at the site of stimulation. The depressed NOD-acute nociceptive and neurogenic inflammatory responses were not due to ongoing autoimmune inflammation, because NOD.*scid* mice, which lack lymphocytes, were not different from NOD (Figures 4E and 4F). Thus, the TRPV1^{NOD} sequence abnormality appears to produce dysfunction of TRPV1-mediated responses to capsaicin.

To assess TRPV1 function more directly, we recorded capsaicin-evoked Ca^{2+} responses in DRG neurons from NOD and NOR mice (Figure 5). The maximum NOD DRG Ca^{2+} response to capsaicin was significantly smaller than that of NOR DRG neurons ($p < 0.01$, Figures 5A–5C). In addition, the maximum capsaicin response was reduced and required 10-fold higher drug concentrations in NOD DRG neurons, compared with that in NOR ($p < 0.05$, Figure 5C). In contrast, KCl-evoked Ca^{2+} responses of NOD and NOR DRG neurons were not different (Figure 5D), indicating that NOD mice do not exhibit a general abnormality in Ca^{2+} responsiveness. The most-direct readouts of TRPV1 function are stimulus-evoked current responses, and we found that capsaicin-evoked whole-cell currents were significantly smaller in DRG neurons from NOD mice as compared with NOR mice (Figure 5E).

Because of the depressed TRPV1 function, we measured TRPV1 protein expression in DRGs and found that the basal TRPV1 protein level in NOD mice was lower than that in NOR (Figure 5F). Thus, the depression of capsaicin-evoked Ca^{2+} and current responses in DRG neurons from NOD mice may in part reflect decreased steady-state expression levels of TRPV1^{NOD}. The rightward shift in the capsaicin concentration-response relationship suggests that the functionality of the TRPV1^{NOD} protein itself may also be reduced as compared with TRPV1$^{wild\text{-}type}$. Collectively, we discovered functional abnormalities in nociceptive behavior, neuropeptide secretion, channel function, and expression that define TRPV1^{NOD} as a hypofunctional mutant.

Localized Pancreatic sP Administration Reverses Islet Pathology

We reasoned that abnormal TRPV1 function might selectively lead to islet pathology if there was a local, disease-predisposing TRPV1 effect on β cell function and if that effect was removed in NOD^{caps} mice. The insulin-rich islet milieu represents a unique environment for TRPV1^{+} nerve terminals, as they express insulin receptors and insulin sensitizes and lowers the activation threshold of TRPV1 channels (Van Buren et al., 2005). Based on the diminished capsaicin-evoked neurogenic inflammation in NOD mice, and the reduced TRPV1 expression and function, we hypothesized that release of neuropeptides from sensory neuron terminals may be depressed in these mice. We examined a prominent neuropeptide, sP (O'Connor et al., 2004), and found that DRG sP levels were elevated

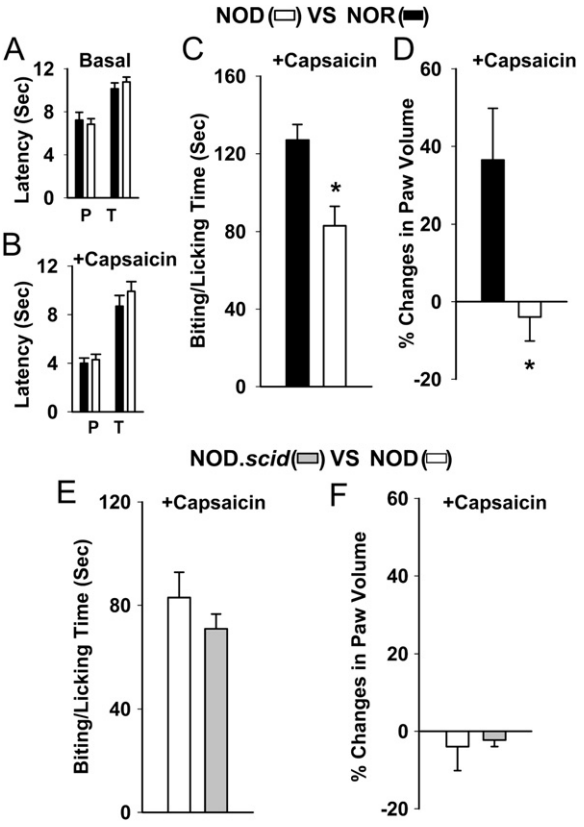

Figure 4. Depressed TRPV1-Mediated Responses in NOD Mice

Basal paw withdrawal (P) and tail flick (T) latencies to heat stimuli for NOD and NOR mice (n = 10/group) (A) and the latencies after capsaicin injection (B). Capsaicin-evoked (0.1 μg/10 μl) biting/licking during the first 5 min after capsaicin injection in NOD and NOR mice (C) or NOD.*scid* and NOD mice (E). Paw volume changes 45 min after capsaicin injection in NOD and NOR mice (D) or NOD.*scid* and NOD mice (F). Error bars represent + one SEM.

in NOD compared with NOR mice (Figure S8). NOD^{ctrl} and NOD.*scid* pancreas shows accumulation of more sP in nerve endings than B6 mice (Figure S2). Overall, the enhanced accumulation of sP is consistent with reduced sP release in NOD mice.

If depressed sP release was critical for NOD islet pathology, then increasing pancreatic sP levels should affect the pathogenic process, and we injected sP via the pancreatic artery. Figure 6A demonstrates the selective delivery of intra-arterially (i.a.) injected Evans Blue dye to pancreatic and pancreatic lymph node tissue (insert). In prediabetic NOD^{ctrl} animals 12–14 weeks of age, we found that within 2 days after i.a. sP injection (2 nmoles/kg), about 80% of all islets were free of T cell infiltration (Figures 6B and 6C) and there were only small, residual infiltrates in the remainder. In animals receiving pancreatic i.a. vehicle injection, only 6% of islets were lymphocyte free ($p < 0.0001$) (Figure 6D). Systemic (i.v.) injection of the same sP dose did not have similar effects (see below, Figure 6I).

Analogous observations were made after pancreatic i.a. injection of sP into newly diabetic NOD mice, 2–3 days after diagnosis. After sP administration, and without insulin therapy, over half of the i.a. injected diabetics normalized blood glucose levels (Figure 6E, red lines). In these fully responsive mice, fasting blood glucose returned to near normal levels rapidly and remained at these levels for 2–8 weeks. Raising pancreatic sP levels dramatically enhanced insulin sensitivity, suggesting that the elevated insulin resistance at diagnosis was normalized (Figure 6F). On average, mice that reversed diabetes had less-extreme hyperglycemia at the time of diagnosis than did the nonresponding mice, likely reflective of a larger residual β cell mass at the time of sP administration. However, even in mice that failed to reverse hyperglycemia (blue lines), i.a. sP caused a significant improvement of metabolic control, preventing the progressive loss of body weight typical of overtly diabetic NOD mice (Figure S10). This improvement corresponds to significantly ($p < 0.0001$) improved insulin sensitivity (Figure 6F, blue line), which enhances the effectiveness of a small remaining β cell mass at diabetes onset. In all vehicle-injected control animals, blood glucose rose progressively, body weights declined, and animals were sacrificed because of severe diabetes between days 12 and 16.

Abundant expression of the NK1R sP receptor has been reported for islet infiltrating lymphocytes (Persson-Sjogren et al., 2005), and therefore, one likely target for sP is activated pancreatic T cells. We detected NK1R expression on a portion of T cells from pancreatic lymph nodes (Figure 6G). Upon in vitro activation with concanavalin A (Con A), essentially all NOD splenic T cells expressed NK1R (Figure 6G, insert). To determine the functional effect of NK1R ligation, we tested the sP response of activated CD4$^+$ NOD T cells in vitro. sP abrogated cell proliferation and survival in a dose-dependent fashion (Figure 6H).

To determine the in vivo effect of pancreatic i.a. sP injection on clonal T cell expansion in pancreatic lymph nodes, we used islet-reactive, BDC2.5 T cell receptor transgenic T cells after labeling with a fluorescent dye, CFSE (Ji et al., 1999). Cells were transferred into 12-week-old normoglycemic NOD females, which had received pancreatic i.a. or systemic i.v. sP (red lines) or vehicle injections 12–16 hr prior (Figure 6I). BDC2.5 T cells from pancreatic lymph nodes were analyzed by flow cytometry 4 days later. Injection of sP reduced cellularity and clonal expansion, measured by dye dilution ($p = 0.003$). Systemic (i.v.) injection of the same sP dose had no effect on expansion of BDC2.5 T cells in pancreatic lymph nodes, suggesting a pancreas tissue-conditioning effect of i.a. pancreas injection that lasts at least 12–16 hr. A third set of animals received BDC2.5 cells that were in vitro pretreated with sP or vehicle overnight. This in vitro pretreatment with sP reduced the ability of these cells to expand in pancreatic lymph nodes (Figure 6I, bottom, $p = 0.0045$). As equal numbers of viable cells were transferred, these observations imply that sP also has a T cell conditioning effect.

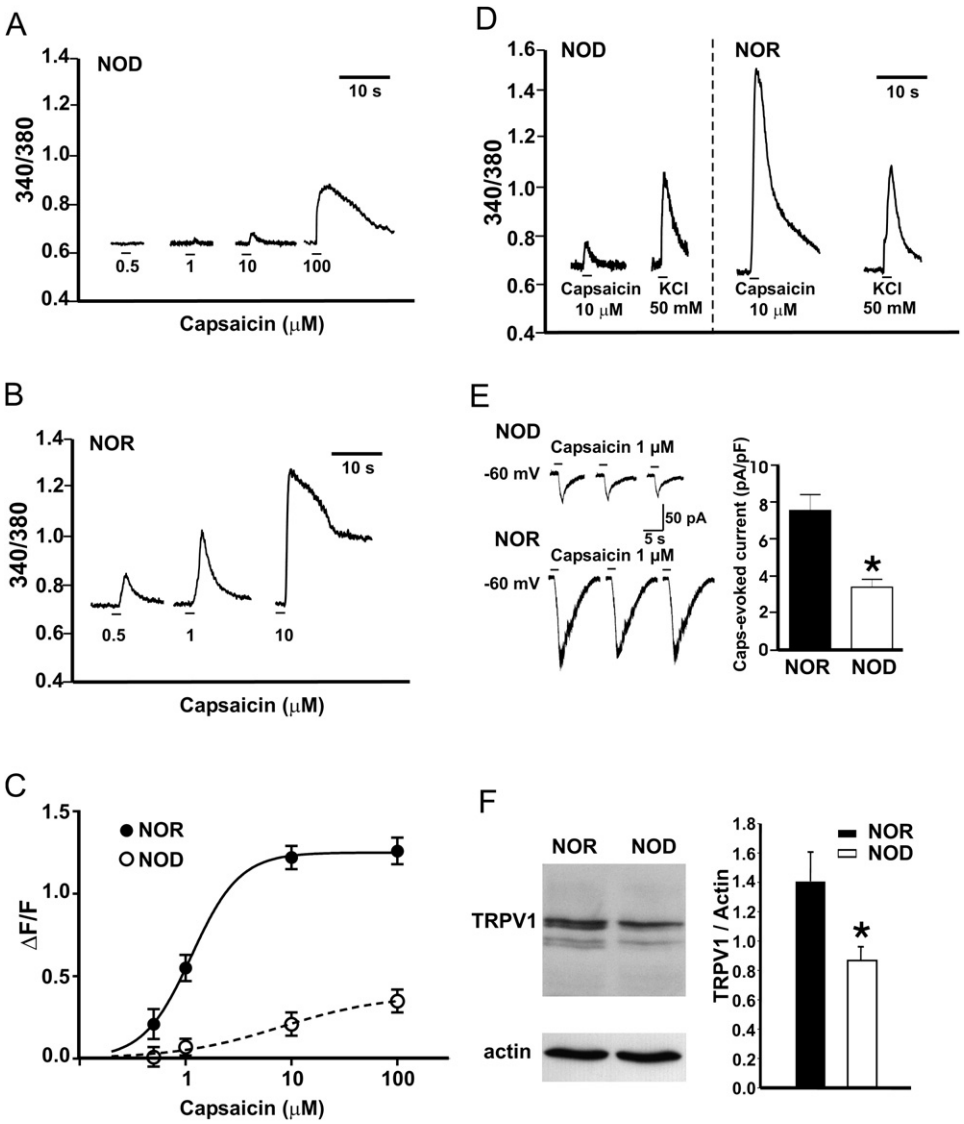

Figure 5. Decreased TRPV1 Function and Expression in NOD DRG Neurons

Capsaicin-evoked Ca^{2+} response in cultured DRG neurons from NOD (A) and NOR mice (B), representative of five neurons/group. Dose-response curve of capsaicin-evoked Ca^{2+} responses in NOD and NOR DRG neurons (n = 4/group) (C). KCl-evoked Ca^{2+} response in NOD and NOR DRG neurons (D). Capsaicin-evoked currents with whole-cell patch-clamp recording from DRG of NOD and NOR mice (n = 5/group, no difference in NOD versus NOR average DRG capacitance) (E). TRPV1 expression in DRG extracts from NOD and NOR mice (n = 6/group) (F). Error bars represent + one SEM.

Taken together, the data suggest that reduced neuropeptide release by pancreatic $TRPV1^{+}$ nerve terminals is a pathogenic event in NOD diabetes amenable to therapeutic correction.

TRPV1 Function and β Cell Stress

β cell stress has previously been suggested as an early element of T1D pathoetiology, and we asked if the hypofunctional $TRPV1^{NOD}$ is related to signs of β cell stress, hyperinsulinism, and abnormal glucose clearance observed even in young NOD mice (Rosmalen et al., 2000; van de Wall et al., 2005). We compared measures of β cell function in untreated and in capsaicin-treated NOD.*scid* mice, and in C57/BL6J ("B6") and *trpv1*$^{-/-}$ mice, the latter with a normal complement of sensory afferent neurons but absent TRPV1 expression (Caterina et al., 2000). NOD.*scid* mice were used to ascertain absence of lymphoid islet infiltrations in NOD experiments, and CD1 mice provided controls (Figure S9).

The high-normal serum glucose levels after i.p. glucose challenge in 10- to 12-week-old NOD.*scid*^{ctrl} mice were significantly reduced in NOD.*scid*^{caps} mice (Figure 7A, p = 0.004). The improved NOD.*scid*^{caps} glucose response was associated with significantly less insulin production

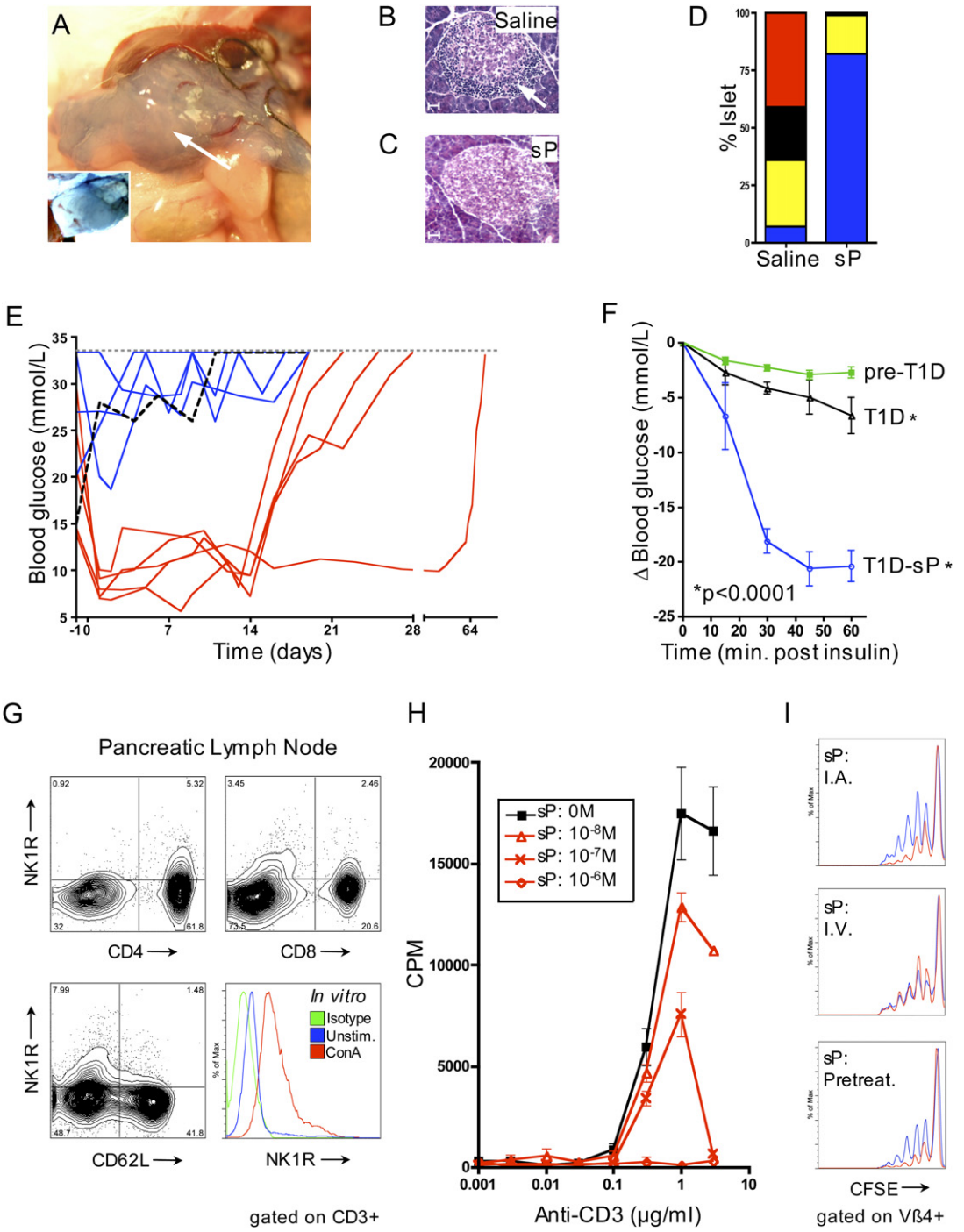

Figure 6. i.a. Pancreas Injection of sP Reverses Islet Pathology

i.a. Evans Blue dye injection selectively stains pancreas (arrow) and pancreatic lymph node (insert) (A). Representative, H&E-stained pancreas sections from prediabetic (12 week) NOD females 2 days after i.a. pancreas injection with saline (B) or sP (C); arrow, insulitis lesions, and scale bars, ~10 μm. Insulitis scores of 250–300 islets from saline- or sP-treated NOD prediabetics (D). Blood glucose levels in newly diabetic mice i.a. injected with saline (black line) or sP; red line, responder mice; blue line, nonresponder mice; each line represents one mouse (E). Insulin sensitivity of prediabetic (green), diabetic (black, i.a. saline injected) animals, and apparent nonresponder mice after i.a. pancreas sP injection (blue line) (F); graph shows changes in blood glucose after i.p. injection of insulin (n = 4/group). Flow cytometric measurement of CD4 and NK1R expression in $CD3^+$ prediabetic NOD lymphocytes; NK1R expression is upregulated after in vitro ConA stimulation (1 mg/ml, bottom) (G). Purified $CD4^+$ T cells from NOD $\beta 2m^{null}$ splenocytes were costimulated with plate-bound anti-CD3 and anti-CD28 in the presence of various concentrations of sP(H); graph shows mean incorporation of H^3-TdR. (I) Clonal expansion, in pancreatic lymph nodes, of CFSE-labeled, $CD4^+$ BDC2.5 T cell receptor

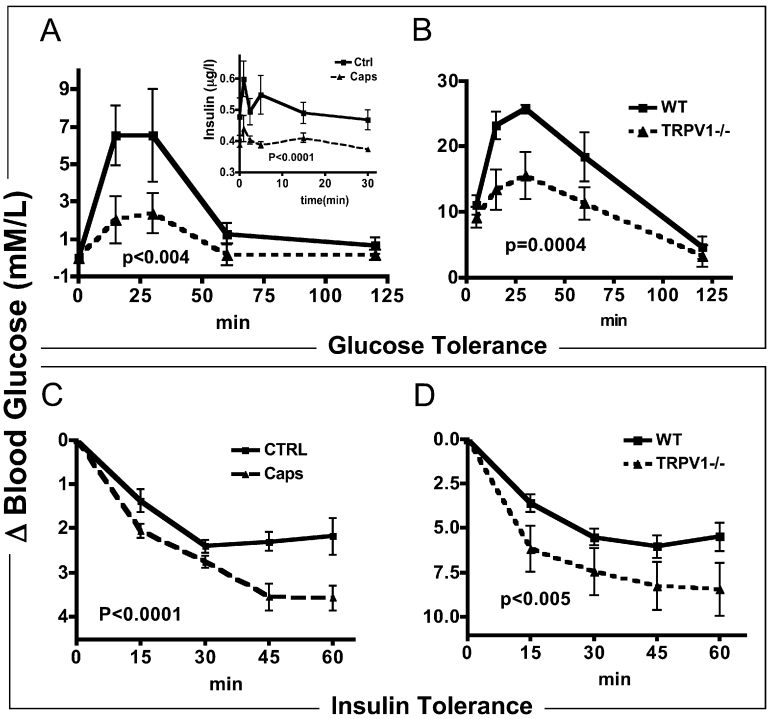

Figure 7. Glucose Tolerance and Insulin Sensitivity in NOD^{caps} and TRPV1^{null} B6 Mice

Changes in blood glucose (mmol/L) (A) and insulin levels (insert) after i.p. glucose challenge (2 g/kg) in fasted NOD^{ctrl}, NOD^{caps} mice, and wild-type or TRPV1^{null} B6 mice (n = 5/group) at 6 to 8 weeks old (B); insert, parallel changes in plasma insulin levels. Insulin sensitivity: changes in blood glucose (mmol/L) after i.p. injection of insulin (Humulin, 0.75 U/kg) in similar groups of mice (C and D). Error bars represent ± one SD.

(Figure 7A, insert), suggesting more-effective insulin action after removal of TRPV1$^+$ sensory neurons.

B6 mice develop elevated insulin resistance and type 2 diabetes-like disease (Parekh et al., 1998), attributed to the functional deletion of nicotinamide transhydrogenase (Freeman et al., 2006). Consistently, we observed high blood glucose levels after standard i.p. glucose challenge (Figure 7B). B6.*trpv1*$^{-/-}$ mice showed a significantly improved glucose response, analogous to NOD^{caps} mice, raising the possibility that TRPV1 may play a general role in β cell physiology.

To more directly assess if both data sets could reflect enhanced insulin sensitivity due to TRPV1 removal, we measured glucose clearance after a single insulin injection. Compared to their respective control animals, NOD^{caps} and B6.*trpv1*$^{-/-}$ mice showed significantly enhanced and accelerated glucose clearance, which we interpreted as evidence for reduced insulin resistance due to the absence of TRPV1 in these two independent animal models. Similar outcomes in NOD^{caps} and B6.*trpv1*$^{-/-}$ mice link the observed effects on β cell function to TRPV1 itself. Enhanced insulin resistance associated with TRPV1^{NOD} constitutes a persistent β cell stress, likely worsening with progressive islet inflammation (Nielsen et al., 2004). TRPV1 and TRPV1$^+$ sensory neurons impact insulin/glucose homeostasis in these models of type 1 and type 2 diabetes.

Congenic Replacement of NOD.Idd4

As a test of our conclusions that TRPV1 plays a fundamental role in islet inflammation and insulin homeostasis, we investigated NOD.*B6.Idd4*-congenic mice (Figure 8). These mice carry wild-type TRPV1 in this locus and are insulitis- and diabetes resistant despite the fact that their splenocytes transfer diabetes to NOD.*scid* mice (Grattan et al., 2002). Consistent with wild-type *trpv1* function, we found that these congenics have normalized behavioral responses to cutaneous capsaicin injection (biting/licking, Figure 8A), neurogenic inflammation after paw injection with capsaicin (Figure 8B), and Ca^{2+} responses in capsaicin-stimulated DRG (Figure 8C). The glucose responses of weaned *Idd4* congenics were comparable to those in NOD control mice; however, there was a significant reduction in glucose-induced insulin secretion, suggesting an absence of elevated insulin resistance in these animals (Figure 8D).

These NOD congenic mice resemble NOD^{caps} mice, as both are insulitis/diabetes protected, although their T cells transfer diabetes to NOD.*scid* recipients. TRPV1^{NOD} adds elevated insulin resistance as a new, strikingly diabetes-relevant phenotype to the NOD *Idd4.1* risk locus, which is presently associated only with insulitis; transgenic rescue experiments will be required for formal proof that *trpv1* is or is not the *Idd4.1* diabetes risk gene.

transgenic (diabetogenic) T cells, 4 days after transfer into saline (blue line) or sP treated (red line), 12-week-old normoglycemic NOD females. TCR-Vβ4$^+$CFSE$^+$ populations from two to five separate experiments are shown; pooled data are presented as mean ± SEM. Error bars represent ± one SD.

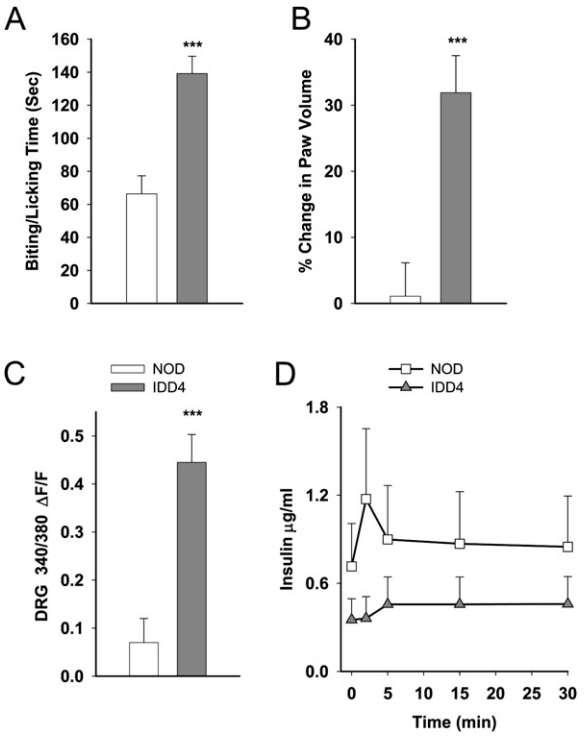

Figure 8. Rescue of TRPV1 Function and Insulin Resistance in NOD.B6.Idd4-Congenic Mice

Capsaicin-evoked (0.1 μg/10 μl) biting/licking during the first 5 min after capsaicin injection in NOD and NOD.*B6.Idd4* mice (n = 6/group) (A). Paw volume changes 45 min after capsaicin injection in NOD (n = 4) and NOD.*B6.Idd4* (n = 6) mice (B). Capsaicin-evoked Ca^{2+} response in cultured DRG neurons from NOD (n = 4 neurons) and NOD.*B6.Idd4* (n = 4 neurons) mice (C). Insulin levels after i.p. glucose challenge (2 g/kg) in fasted NOD and NOD.*B6.Idd4* mice (n = 5/group) (D). Error bars represent ± one SEM.

DISCUSSION

In the different, independent animal strains and experimental conditions analyzed, TRPV1 emerges as a central controller of both islet stress and T cell infiltration. Elimination of $TRPV1^+$ neurons by capsaicin, transient functional normalization by acute local sP injection, or replacement with wild-type *trpv1* in *Idd4* congenics has the same, islet-specific outcome: normalized insulin sensitivity and abrogation of insulitis, despite unimpeded generation of autoreactive lymphocytes that can transfer disease to untreated NOD hosts. The most parsimonious explanation unifying these observations is a local feedback interaction between β cells and the primary sensory neurons innervating islets (Figure S11), with nerve terminals responding to local insulin with release of neuropeptides that sustain β cell physiology in an optimal range. Normally, this interaction is in balance, but in the NOD mouse, hypofunction of TRPV1 unbalances the feedback, with β cell stress due to hyperinsulinism, insulin resistance, and infiltration by autoreactive T cell pools independently generated in the NOD mouse. Removing TRPV1 neurons leads to elimination of the unbalanced, pathogenic interaction, whereas administering sP exogenously may renormalize the interaction transiently.

A direct neuropeptide effect on β cells has previously been reported, with deleterious outcomes at low concentrations but β cell augmenting effects at higher concentrations (Barakat et al., 1994; Bretherton-Watt et al., 1992; Hermansen and Ahren, 1990). Our hypothesis, based on the foregoing results that in NOD mice suppressed neuropeptide secretion is a pathogenic event, was positively answered through two independent approaches: removal of $TRPV1^+$ neurons and local i.a. pancreas injection with sP, both with similar results. Pancreas sP injection normalized all parameters tested: clearing of insulitis lesions, enhancement of insulin sensitivity, and consequent reversal of overt diabetes that lasted for weeks. The only other strategy to reverse NOD diabetes is toxic immunosuppression with anti-CD3 antibodies, now also in clinical trials with human diabetics (Keymeulen et al., 2005).

Collectively, our findings are inconsistent with the view that diabetes is due solely to immunological and endocrine abnormalities. Rather, our observations demonstrate that the nervous system, in particular $TRPV1^+$ primary afferent neurons, has a critical role in diabetes pathoetiology. Analogous findings in NOD^{caps}, NOD.*Idd4* congenics, and *trpv1* knockout mice add strength to our conclusions, as does an earlier report demonstrating that another TRPV1-dependent neuropeptide, CGRP, prevents diabetes when transgenically overexpressed in the islet (Khachatryan et al., 1997). Recently, we generated preliminary evidence for insulitis- and diabetes protection by transsection of sensory nerves innervating the pancreas (J.Y., Y.C., H.T., L.T., R.R., M.W.S., and H.-M.D., unpublished data), providing yet another line of support for the role of $TRPV1^+$ sensory neurons in T1D pathoetiology.

The mapping of several NOD disease-associated phenotypes to a single, mutant protein, TRPV1, implies that $TRPV1^+$ sensory afferents are key elements for normal islet physiology, opening broad new areas of research, including insulin resistance (Moesgaard et al., 2005), which remains a challenge after decades of intense investigation (LeRoith and Gavrilova, 2006).

Our data allowed us to identify the molecular mechanism that translates a system-wide genetic *trpv1* defect into pancreas-specific disease. $TRPV1^+$ sensory neurons express high-affinity insulin receptors, and insulin potentiates TRPV1 currents (Van Buren et al., 2005) and lowers TRPV1 thermal activation thresholds (Sathianathan et al., 2003). At body temperature, the insulin-rich islet milieu should generate tonic TRPV1 current activation with associated neuropeptide release impacting on basal insulin secretion, a local control circuit first envisioned over a decade ago (Hermansen and Ahren, 1990). In NOD mice, this sensory nerve terminal-β cell circuit has gone astray, with disease prevention through either its removal or through local supply of sufficient neuropeptide.

We demonstrated that $TRPV1^+$ sensory afferents control pancreatic tissue access for immune cells, which

may occur through modifying their immigration, residence, emigration, or a combination of these elements. It is likely that progressive islet infiltration will also compound β cell stress, which we believe is central to T1D pathoetiology. There is human disease precedence for a role of sensory neurons controlling lymphocyte tissue access, because rare patients without sensory nerves (CIPA syndrome) succumb to massive infections with little tissue infiltration, despite normal in vitro immune functions (Indo et al., 1996).

We discovered mutations in the coding sequence of TRPV1^{NOD} gene contained within the *Idd4* diabetes risk locus (Grattan et al., 2002; Ivakine et al., 2005; McAleer et al., 1995). NOD.*B6.Idd4* congenic mice show normalized behavioral, electrophysiological, and insulin-resistance phenotypes. Intriguingly, the *TRPV1* locus is contained within other overlapping autoimmune loci (*eae7*, *orch3*, and *streptozotocin sensitivity*) (Babaya et al., 2005; Butterfield et al., 1999; Butterfield et al., 1998), raising the possibility that TRPV1 may play a role in other autoimmune conditions. Indeed, B6 mice, relatively resistant to streptozotocin-induced T1D, show increased streptozotocin susceptibility in B6.*trpv1*$^{-/-}$ mice (data not shown).

Collectively, our findings identify TRPV1^{+} sensory neurons as important elements of diabetes pathoetiology, with effects that provide rational mechanisms of the tissue selectivity of the disease, its links to β cell physiology, stress, and insulin resistance. Our observations open new avenues for therapeutic strategies, raising the possibility that sensory nerve dysfunction may contribute to prediabetes initiation and progression in diabetes-prone humans.

EXPERIMENTAL PROCEDURES

Mice

All mice were obtained from the Jackson Laboratories (Bar Harbor, Maine) and maintained under approved protocols in our vivarium (NOD female T1D incidence: 85%–90%). In adoptive transfer experiments, splenocytes from three to five donors indicated were pooled and 10^7 viable cells/mouse were injected (100 μl i.v.) into irradiated (300 rad) recipients. Diabetes was defined as diabetic blood glucose measurements (>13.8 mM/l) on two consecutive days.

T Cell Studies

Splenocytes (4 × 10^5 cells/well) or lymph node cells (2 × 10^5 lymph node cells plus 2 × 10^5 irradiated [1100 rad], syngeneic splenocytes/well) from 3- to 24-week-old NOD females were cultured in AIM V serum-free medium containing T1D target antigens (0.002–50 μg/ml) as described (Winer et al., 2003). [3H]thymidine incorporation was measured after 72 hr. To normalize pooled data, we calculated a stimulation index (SI, cpm antigen stimulated/medium control). In some proliferation studies, plate-bound anti-CD3 (0.001–3 μg/m) and anti-CD28 (0.2 μg/ml) (BD PharMingen) were used to stimulate CD4^{+} T cells negatively selected from NOD β2m^{null} splenocytes.

i.a. Pancreas Injection

The aorta was developed with minimal trauma and ligated just prior to the celiac branching and injected (32G needle) with Evans Blue (3 mg/kg/100 μl; Sigma), sP (2 nmol/100 μl; Sigma), or saline. Ligations were released after closure of the injection site.

5- and 6-Carboxyl-Fluorescien Succimidyl Ester Labeling

For dye dilution in vivo clonal expansion studies, splenic CD4^{+} T cells from NOD-BDC2.5 females were incubated with 2.5 μM CFSE (10′/37°C, Molecular Probes, Eugene, OR) in PBS. Prediabetic (12 week) NOD females pretreated 12 hr prior with sP or saline were injected i.v. with 3 × 10^6 CFSE-labeled CD4^{+} T cells.

Immunofluorescence and Histology

Frozen murine pancreas sections were fixed in 4% paraformaldehyde, blocked, and stained with variously fluorescence- or biotin-conjugated antibodies or strepavidin as described (Winer et al., 2003). TRPV1 staining was performed on snap-frozen sections of NOD female pancreas with an overnight incubation of primary antibody at 4°C. To score insulitis severity, pancreata were fixed in 10% buffered formalin for a minimum of 24 hr. Histological sections were stained with hematoxylin and eosin and scored by three blinded observers (Winer et al., 2003). NRP-V7/H-2K^{d} tetramer positivity was analyzed on gated CD8^{+}B220^{-} cells and reported as percentage of cells binding the tetramer minus the percentage of cells binding the negative control tetramer TUM/H-2K^{d}.

Molecular Cloning

PCR amplification used TRPV1-specific primers with cDNA from NOD, NOR, and B6 DRG, cloning followed standard procedures using the TOPO XL PCR Cloning Kit (InVitrogen, Mississauga, ON).

Behavioral Studies and Paw Volume Measurement

All behavioral tests of the mouse strains indicated were conducted between 9 and 16 hr after appropriate acclimatization to the testing environment and with standard intrumentation/apparatus. Paw volume measurements used a plethysmometer (Ugo Basile), and values were standardized as a percentage of individual preinjection volumes.

Ca^{2+} Response Measurement

DRG from donors indicated were isolated and cultured 3–5 days in F12 medium with 10 ng/mL nerve- and 10 ng/mL glial-derived NGF. Fura-2 (Molecular Probes, Eugene, OR) was used to assess $[Ca^{2+}]_i$ by ratiometric measurement. Each 340 nm image was divided, on a pixel-by-pixel basis, by the corresponding 380 nm image, producing a ratio. Averaged values of the ratios within each region of interest were plotted as a function of time. Whole-cell DRG patch-clamp recordings were performed at room temperature. TRPV1 currents were recorded using an Axopatch 1-D amplifier, data were digitized with DigiData1322, filtered (2 kHz), and acquired by the pClamp9.0 program. Recordings in which the series resistance varied by more than 10% were rejected.

Immunoblotting

DRG or dorsal horns (spinal cord) were dissected and snap-frozen. Extracted total protein (45 μg DRG protein, 30 μg spinal cord) was electrophoresed (10% acrylamide gels), western blotted, probed overnight with rabbit anti-TRPV1 antibody (1:250, Oncogene), and developed with the ECL kit (Amersham).

Statistics

All tests were two tailed, significance was set at 5%. Life tables, t tests (flow cytometry), ANOVA, and Fisher's exact test were used as described in the text.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and eleven figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/6/1123/DC1/.

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