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The p75 Neurotrophin Receptor Is Required for the Major Loss of Sympathetic Nerves From Islets Under Autoimmune Attack

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Our goal was to determine the role of the p75 neurotrophin receptor (p75NTR) in the loss of islet sympathetic nerves that occurs during the autoimmune attack of the islet. The islets of transgenic (Tg) mice in which β -cells express a viral glycoprotein (GP) under the control of the insulin promoter (*Ins2*) were stained for neuropeptide Y before, during, and after virally induced autoimmune attack of the islet. *Ins2-GP^{Tg}* mice injected with lymphocytic choriomeningitis virus (LCMV) lost islet sympathetic nerves before diabetes development but coincident with the lymphocytic infiltration of the islet. The nerve loss was marked and islet-selective. Similar nerve loss, chemically induced, was sufficient to impair sympathetically mediated glucagon secretion. In contrast, LCMV-injected *Ins2-GP^{Tg}* mice lacking the p75NTR retained most of their islet sympathetic nerves, despite both lymphocytic infiltration and development of diabetes indistinguishable from that of p75NTR wild-type mice. We conclude that an inducible autoimmune attack of the islet causes a marked and islet-selective loss of sympathetic nerves that precedes islet collapse and hyperglycemia. The p75NTR mediates this nerve loss but plays no role in mediating the loss of islet β -cells or the subsequent diabetes. p75NTR-mediated nerve loss may contribute to the impaired glucose counterregulation seen in type 1 diabetes.

Two neuropathies associated with diabetes are well-recognized: diabetic autonomic neuropathy (1–3) and

somatosensory neuropathy (4,5). Their multiple mechanisms have been linked to chronic hyperglycemia (6,7) in a unifying hypothesis (8). There is also less extensive evidence for acute damage to sensory (9) and sympathetic (10,11) innervation supplying the islet. This mechanism may involve insulin deficiency instead of hyperglycemia. Sympathetic defects may contribute to the impaired glucagon response to hypoglycemia seen early in type 1 diabetes (12), since activation of pancreatic sympathetic nerves stimulates glucagon secretion (13–15), and hypoglycemia activates these nerves (16,17).

Since the glucagon response to insulin-induced hypoglycemia depends both on relief from tonic inhibition by the islet β -cell (18) and active stimulation by the autonomic nervous system (19), defects in both have been proposed as causes of this impairment (18,19). One autonomic defect, which we named early sympathetic islet neuropathy (eSIN), is present in diabetic BB rats (20), NOD mice (21,22), and type 1 diabetic humans (23). This marked loss of islet sympathetic nerves is sufficient to impair the glucagon response to sympathetic activation (21,24). Since eSIN is not present in either chemically induced diabetes (20,21) or in type 2 human diabetes (23), it is likely triggered by the immune attack on the islet, a hypothesis that was strengthened by finding a strong correlation between invasive insulinitis and the loss of islet sympathetic nerves in NOD mice (21).

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The studies above established the unique characteristics of eSIN. The first is its early onset: eSIN occurs as early as 5 days after diabetes onset in BB rats (20) and sometimes even before diabetes presentation in NOD mice (21). The second is its severity: 85% of islet sympathetic nerves are lost in diabetic BB rats (20), 66% in diabetic NOD mice (21), and 93% in type 1 diabetic patients (23). The third is its islet selectivity: there is no loss of sympathetic nerves from the surrounding exocrine pancreas (20,23). Such localized pruning of sympathetic axons also occurs in the uterus during estrus (25) and during development of target innervation (26). Importantly, the latter study demonstrated a segmental axonal degeneration secondary to activation of the p75 neurotrophin receptor (p75NTR) on sympathetic axons (26). Thus, we hypothesize that, during the autoimmune attack of the islet, invading lymphocytes either secrete an activating ligand for p75NTR or stimulate islet cells to do so.

To determine the involvement of the p75NTR in eSIN, we took advantage of a transgenic (Tg) model of immune-mediated diabetes, the insulin promoter (*Ins2*)-GP^{Tg} mouse (27), in which the immune attack of the islet could be induced on demand, in contrast to other animal models of naturally occurring autoimmune diabetes (i.e., the BB rat or NOD mouse). We also needed a model in which the islets of nondiabetic controls had no nerve loss, which ruled out NOD mice (21). Finally, to delete *Ngfr*, the gene for p75NTR, by cross-breeding, we needed a model of immune-mediated diabetes on the same genetic background as p75NTR knockout (KO) mice (C57Bl/6), again ruling out the NOD mouse. The *Ins2*-GP^{Tg} mouse fulfills all these requirements for studying the mechanism of islet nerve loss. Although there are differences in the onset and severity of autoimmune diabetes between humans and any of the animal models above, they all display a marked loss of islet β -cells, presumably due to T lymphocytes, and they all display a marked loss of islet sympathetic nerves. Thus, the mechanism for the loss of islet sympathetic nerves in the *Ins2*-GP^{Tg} mouse may be similar to those in the other animal models above and in human type 1 diabetes.

Injection of lymphocytic choriomeningitis virus (LCMV) into *Ins2*-GP^{Tg} mice produces a systemic viral infection, which initiates a T-lymphocyte-mediated attack on the circulating virus owing to an antigenic glycoprotein (GP) on its envelope. Because the islet β -cells of these mice transgenically express this viral GP, lymphocytes also aggressively infiltrate the islet, followed by β -cell destruction, ultimately leading to diabetic hyperglycemia (27). The precise timing and separation of these events is an advantage in this model because it allows determination of their respective contributions to the loss of islet sympathetic nerves. The presence of live virus is a disadvantage in this model because it creates a biosafety level 2 (BSL2) biohazard, which precludes sophisticated *in vivo* studies of the functional impact of this nerve loss on glucagon secretion.

Using the *Ins2*-GP^{Tg} model, we addressed five major questions related to the loss of islet sympathetic nerves. First, does this Tg model of immune-mediated diabetes lose the majority of its islet sympathetic nerves like those of naturally occurring models of autoimmune diabetes? Second, is this degree of nerve loss sufficient to impair glucagon secretion? Third, is the onset and islet selectivity of the nerve loss similar to that seen in naturally occurring autoimmune diabetes? Fourth, what is the contribution of viral infection, lymphocytic infiltration of the islet, and diabetic hyperglycemia to the loss of islet sympathetic nerves? Fifth, and most importantly, is the p75NTR required for this loss of islet sympathetic nerves?

RESEARCH DESIGN AND METHODS

Animals

Ins2-GP^{Tg} Mice

Ins2-GP^{Tg} mice on a C57Bl/6 background were bred in a specific pathogen-free facility, and offspring were genotyped at 4 weeks of age using forward and reverse primers for GP (The Jackson Laboratory, Bar Harbor, ME). Male or female *Ins2*-GP^{Tg} mice 8–10 weeks old were injected subcutaneously with LCMV in a BSL2 cabinet. Thereafter, each mouse was placed back into an individually ventilated cage (TECNIPLAST, Buguggiate, Italy) using enhanced BSL2 procedures.

LCMV-infected mice were housed for up to 4.5 additional weeks (3 weeks after turning diabetic). Blood glucose was monitored by a 1 μ L tail vein blood sample (OneTouch Ultra 2; LifeScan, Milpitas, CA) every 2 to 3 days to confirm the development of diabetes (>350 mg/dL; usually 9 days after LCMV treatment). An insulin pellet (Linbit, ~0.1 unit/day; Linshin, Scarborough, ON, Canada) was implanted subcutaneously to prevent ketoacidosis while still allowing marked hyperglycemia. Fifteen groups of *Ngfr*^{+/+} *Ins2*-GP^{Tg} mice or controls (Table 1) were killed for measurement of islet sympathetic nerve area, islet area, invasive insulinitis, ganglionic mRNA, or stimulated glucagon secretion.

Ngfr^{-/-} *Ins2*-GP^{Tg} Mice

Ngfr^{+/-} mice on a C57Bl/6 background (The Jackson Laboratory) were crossbred with *Ins2*-GP^{Tg} mice. Offspring that were positive for GP and heterozygous for *Ngfr* were backcrossed to produce *Ngfr*^{-/-} *Ins2*-GP^{Tg} mice. The *Ngfr* genotype of the offspring was determined using three primers (The Jackson Laboratory). Six groups of *Ngfr*^{-/-} *Ins2*-GP^{Tg} were used for measurement of islet sympathetic nerve area, invasive insulinitis, and islet area or ganglion mRNA (Table 1).

Research involving animals was conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, all protocols were approved by the Institutional Animal Care and Use Committee of the Seattle VA Puget Sound Health Care System, and all mice included in these studies were certified healthy by the Veterinary Medical Officer.

Table 1—Genotype and treatment of the 21 groups used in this study

Groups	Age (weeks)	Treatment (time after)	Diabetes (duration)	Measure	Genotype		N
					Ins2-GP ^{Tg}	Ngfr	
p75NTR WT							
1	10	—	—	NPY	+/?	+/+	7
2	10–11	LCMV 4 days	—	NPY	+/?	+/+	8
3	11	LCMV 7 days	—	NPY	+/?	+/+	8
4	14–15	LCMV 4 to 5 weeks	3 weeks	NPY	+/?	+/+	6
5	14–15	—	—	NPY	+/?	+/+	6
6	14	—	—	NPY	-/-	+/+	8
7	10–17	—	—	mRNA	+/?	+/+	9
8	9	—	—	Th	+/?	+/+	7
9	9	LCMV 7 days	—	Th	+/?	+/+	7
10	13–15	—	—	Th	+/?	+/+	3
11	13–15	LCMV 4 to 5 weeks	3 weeks	Th	+/?	+/+	3
12	10	—	—	NPY	+/?	+/+	6
13	10	6-OHDA 1 week	—	NPY	+/?	+/+	6
14	17–18	—	—	IRG, NE	+/?	+/+	8
15	18–19	6-OHDA 1 week	—	IRG, NE	+/?	+/+	5
p75NTR KO							
1	10	—	—	NPY	+/?	-/-	6
2	11	LCMV 7 days	—	NPY	+/?	-/-	6
3	14–15	LCMV 4 to 5 weeks	3 weeks	NPY	+/?	-/-	6
4	14–15	—	—	NPY	+/?	-/-	6
5	14–15	—	—	NPY	-/-	-/-	8
6	10–14	—	—	mRNA	+/?	-/-	7

IRG, glucagon; NE, norepinephrine; WT, wild type.

Measurement of Ganglionic Ngfr mRNA

Two groups of mice (Table 1) were deeply anesthetized (sodium pentobarbital; 90 mg/kg i.p.) to access the superior cervical ganglion (SCG) and the celiac ganglion (CG). The SCG was excised above the bifurcation of the carotid artery. The CG was excised at the junction of the celiac artery and abdominal aorta. Both ganglia were placed in RNA later, with 1% eosin to dye the ganglia for visualization.

Thawed ganglia were ground with a glass-on-glass pestle until the dyed ganglia were no longer visible. The mRNA was isolated on a spin column (RNeasy; Qiagen), eluted, and then reverse-transcribed (Applied Biosystems). The resultant cDNA was divided into fourths and amplified by a real-time PCR to determine the threshold cycle (C_T) for Ngfr, tyrosine receptor kinase A (TrkA), tyrosine hydroxylase (Th), and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) using Applied Biosystems kits Mm01309637 [m1](#), Mm01219406 [m1](#), Mm00447557 [m1](#), and 4308313, respectively.

Measurement of Islet Area, Nerve Area, and Invasive Insulinitis

Mouse pancreas was fixed in situ, harvested, and prepared for immunohistochemistry as previously described (21). Immunohistochemical staining for Hoechst, neuropeptide Y (NPY), Th, and glucagon and the quantification of islet sympathetic nerve area were done as previously described (21). Invasive insulinitis was semiquantified by Hoechst staining (score 1–6) where 1 is none, 1.5 is possible, 3 is regional, and 6 is full. T lymphocytes were stained using an anti-CD3 antibody (1:5,000; Abcam, Cambridge, MA). T-lymphocytic infiltration was quantified as CD3 area/islet area.

Measurement of the Glucagon Response to Tyramine

Ins2-GP^{Tg} mice, free of LCMV, were injected with 6-hydroxydopamine (6-OHDA; 100 mg/kg i.p.) to mimic the loss of islet sympathetic nerves seen in Ins2-GP^{Tg} mice 7 days after LCMV. One week later, vehicle- and 6-OHDA-treated mice were anesthetized with isoflurane (1.5–2.0%) and placed on a heating pad. Through a midline laparotomy, both the portal vein and inferior vena cava were catheterized. The tip of the portal catheter was advanced to the hilus of the liver to sample glucagon-rich blood. Drugs and replacement blood were infused into the inferior vena cava. After a 45-min stabilization period, portal blood samples were drawn before, during, and after a 5-min tyramine infusion (1,280 μ g/kg/min i.v.). The volume of all blood samples was fully replaced by a concomitant infusion of heparinized donor blood. Plasma glucagon and norepinephrine concentrations were measured using assay methods previously described (21).

Statistics

When comparing data across three groups, we used ANOVA and a post hoc Scheffe test. When making comparison between two groups, we used a two-sample *t* test. All data are expressed as mean \pm SEM.

RESULTS

Loss of Islet Sympathetic Nerves in Ins2-GP^{Tg} Mice

Induction of Autoimmune Diabetes

To validate a model for eSIN in which we could precisely time the induction of autoimmune diabetes, we injected

10-week-old Ins2-GP^{Tg} mice with LCMV and sequentially measured tail vein blood glucose levels. Prior to the LCMV injection, fed glucose levels averaged 175 ± 10 mg/dL ($n = 6$). Diabetic hyperglycemia presented 9 ± 0.2 days after LCMV injection (blood glucose level 492 ± 28 mg/dL; $P < 0.01$). Thereafter, 3 weeks of mild insulin treatment was begun (see RESEARCH DESIGN AND METHODS), sufficient to prevent wasting (Δ body weight 1.9 ± 0.5 g), without significantly lowering diabetic hyperglycemia (blood glucose level 480 ± 46 mg/dL). As expected, islet area in the 3-week diabetic mice ($5,776 \pm 536 \mu\text{m}^2$) was markedly lower than that of the age-matched nondiabetic mice ($21,584 \pm 3,538 \mu\text{m}^2$; $P = 0.0006$). Thus, LCMV injection into Ins2-GP^{Tg} mice induced a severe immune-mediated diabetes that presented precisely 9 days later. Three weeks thereafter, the islets were collapsed, suggesting a major loss of β -cells, and the marked hyperglycemia was sustained despite mild insulin treatment.

Selective Loss of Islet Sympathetic Nerves After Diabetes

To determine if this virally induced Tg model of autoimmune diabetes exhibited the same marked loss of islet sympathetic nerves seen in two non-Tg models of spontaneous autoimmune diabetes (20,21) we quantified islet sympathetic nerve area. The NPY nerve area in islets of 3-week diabetic Ins2-GP^{Tg} mice (Fig. 1C) was 79% lower (Fig. 1E; $P = 0.0002$) than in the islets of their nondiabetic, age-matched controls (Fig. 1A; both $n = 6$).

We next demonstrated that this loss of sympathetic nerves in Ins2-GP^{Tg} diabetic mice was selective for the islet. NPY nerve area in the exocrine pancreas of 3-week diabetic mice (Fig. 1D) was not decreased (Fig. 1F) compared with that of age-matched nondiabetic controls (Fig. 1B).

Loss of Islet Sympathetic Nerves Before Diabetes: Relation to Lymphocytic Infiltration

Because the onset of diabetes was so precise in LCMV-injected Ins2-GP^{Tg} mice, we were next able to determine if islet sympathetic nerves were lost during the autoimmune attack of the islet that precedes the development of diabetic hyperglycemia. Therefore, we examined islet sympathetic nerves before and 4 and 7 days after LCMV injection. Four days after LCMV injection, the sympathetic innervation of the islet appeared normal (Fig. 2C and H). However, 7 days after LCMV injection, there was a marked loss ($\Delta = -59\%$; $P = 0.0002$) of islet sympathetic nerves ($n = 8$; Fig. 2E and H) compared with non-injected, age-matched (10-week) controls ($n = 7$; Fig. 2A and H). Islet area in 7-day LCMV-injected mice ($35,960 \pm 3,526 \mu\text{m}^2$) was not significantly different from non-injected mice ($29,800 \pm 1,120 \mu\text{m}^2$).

To determine if the loss of islet sympathetic nerves seen 7 days after LCMV injection was related to the lymphocytic infiltration of the islet, in separate groups of mice, we stained pancreases with antibodies against CD3 to determine the percent of islet area occupied by T lymphocytes.

Islets were not significantly infiltrated 4 days after the LCMV injection (Fig. 2D and G) but were markedly infiltrated 7 days after LCMV (Fig. 2F and G). Thus, infiltration of the islet by T lymphocytes is associated with a marked loss of islet sympathetic nerves that precedes both diabetic hyperglycemia and islet collapse.

Effect of Islet Nerve Loss to Decrease Glucagon Secretion

To gain insight into the functional consequences of nerve loss similar to that of Ins2-GP^{Tg} mice 7 days after LCMV, we injected a dose of 6-OHDA (100 mg/kg) into Ins2-GP^{Tg} mice to approximate the nerve loss seen 7 days after LCMV. The 6-OHDA injection decreased islet NPY nerve area by 48% (Fig. 3A) versus the 59% loss seen 7 days after LCMV. To determine the impact of this nerve loss on neurotransmitter release and therefore glucagon secretion, we administered tyramine to both control and 6-OHDA-treated mice and measured their portal venous norepinephrine and glucagon responses, both of which were markedly impaired (Fig. 3B and C). Thus, the degree of nerve loss seen in LCMV-treated Ins2-GP^{Tg} mice is sufficient to markedly impair glucagon responses.

Retention of Islet Sympathetic Nerves in Ngfr^{-/-} Mice

Deleting p75NTR From Pancreatic Sympathetic Neurons

In the CG of Ngfr^{+/+} mice, the C_T for Ngfr mRNA was similar to that for TrkA, Th, and the housekeeping gene Gapdh (open bars in Fig. 4B), demonstrating abundant message for both p75NTR and TrkA synthesis in the sympathetic neurons projecting to the pancreas (28) of wild-type mice. Likewise, in the SCG, the C_T for Ngfr mRNA was similar to those for TrkA, Th, and Gapdh (open bars in Fig. 4C).

Genotyping demonstrated the deletion of Ngfr, the gene for p75NTR, from Ins2-GP^{Tg} mice (lanes 3 and 9 in Fig. 4A), which was confirmed by the absence of Ngfr mRNA in both CG (Fig. 4B) and SCG (Fig. 4C). In CG, the deletion of the p75NTR did not change the expression of TrkA, Th, or Gapdh (compare hatched to open bars in Fig. 4B). In contrast, in the SCG of Ngfr^{-/-} mice, there was a small but significant ($P < 0.01$) increase of expression in all three genes (lower C_T in hatched vs. open bars in Fig. 5C).

No Effect of Deleting p75NTR on Islet Sympathetic Nerves Before LCMV Injection

To verify that deleting the p75NTR did not affect the basal sympathetic innervation of the islet, we demonstrated that islet sympathetic nerve area in 14-week-old Ngfr^{-/-} mice ($45 \pm 5 \mu\text{m}^2$ NPY/islet) was comparable to that of Ngfr^{+/+} mice ($43 \pm 5 \mu\text{m}^2$ NPY/islet; both $n = 8$; $P =$ not significant).

No Effect of Deleting the p75NTR on Islet Infiltration or Diabetic Hyperglycemia

To verify that deleting the gene for the p75NTR did not affect islet infiltration or diabetes development, we quantified invasive insulinitis by CD3 staining 7 days after

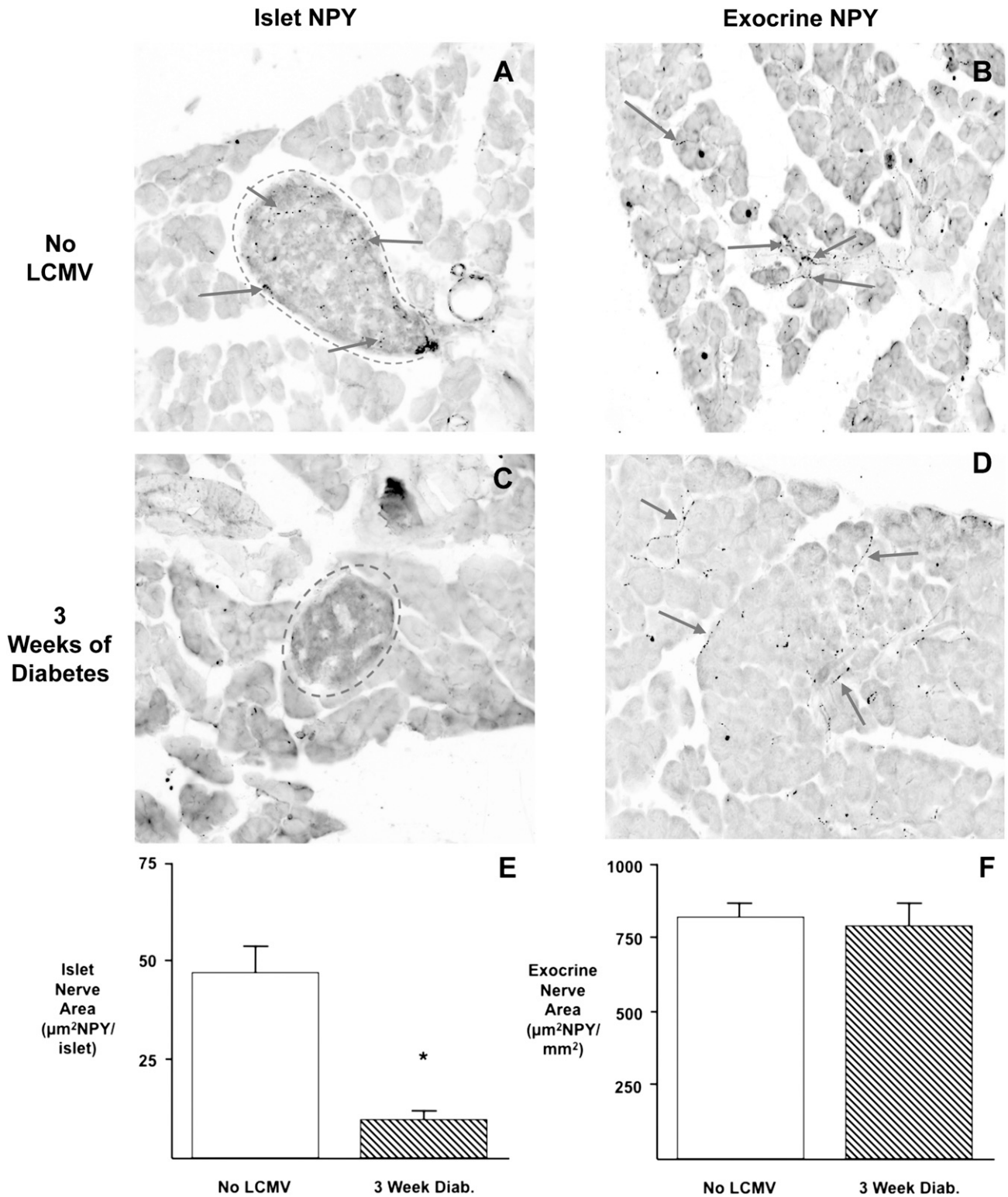


Figure 1—Loss of islet, but not exocrine, sympathetic nerves after 3 weeks of diabetes in LCMV-injected $\text{Ins2-GP}^{\text{Tg}}$ mice. NPY-positive nerve fibers (arrows) in an islet (A) or in the exocrine pancreas (B) of an $\text{Ins2-GP}^{\text{Tg}}$ mouse not injected with LCMV. NPY-positive nerve fibers (arrows) in the exocrine pancreas (D) but not in the islet (C) of an $\text{Ins2-GP}^{\text{Tg}}$ mouse after 3 weeks of LCMV-induced diabetes. NPY nerve area in the islets (E) or exocrine pancreas (F) of $\text{Ins2-GP}^{\text{Tg}}$ mice either not injected with LCMV or after 3 weeks of LCMV-induced diabetes (3 Week Diab.). *Significant difference ($P = 0.0002$) compared with no LCMV control.

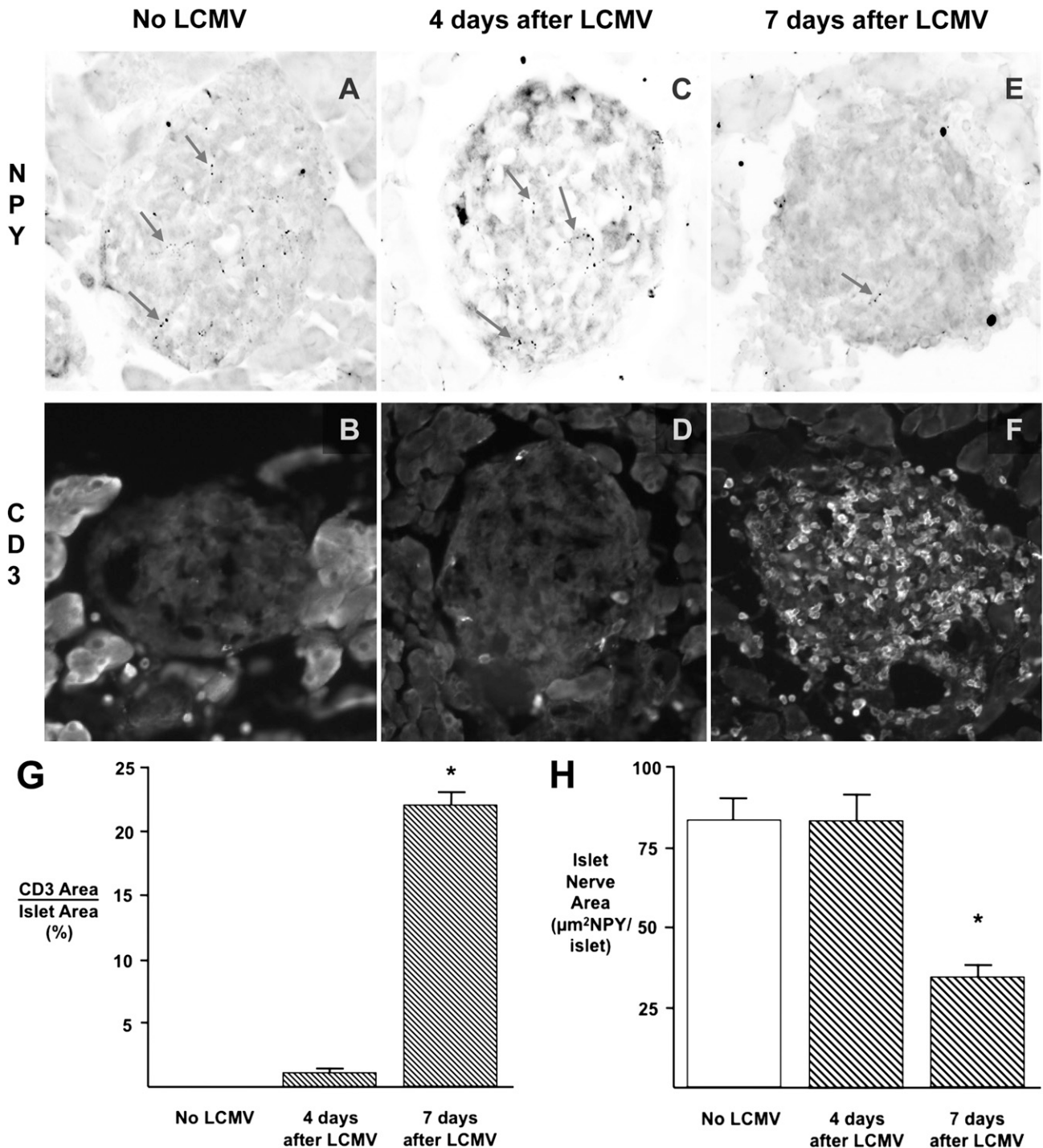


Figure 2—Loss of islet sympathetic nerves is associated with T-lymphocytic infiltration. Loss of NPY nerves (arrows) from an islet of an Ins2-GP^{Tg} mouse 7 (E), but not 4 (C), days after LCMV injection compared with no LCMV control (A). CD3 staining revealing T-lymphocytic infiltration of an islet of an Ins2-GP^{Tg} mouse 7 (F), but not 4 (D), days after LCMV injection compared with no LCMV control (B). Quantification of the islet area occupied by CD3-positive T-lymphocytes (G) or NPY-positive nerves (H) before and 4 and 7 days after LCMV injection. *Significant difference ($P < 0.0005$) compared with no LCMV control.

LCMV and measured glucose levels before, during, and after the presentation of diabetes in *Ngfr*^{-/-} Ins2-GP^{Tg} mice and compared it to those of *Ngfr*^{+/+} Ins2-GP^{Tg} mice. Deleting the gene for the p75NTR had no effect on: 1) invasive insulinitis (Fig. 5A); 2) glucose levels before injection

of LCMV (183 ± 14 vs. 177 ± 6 mg/dL); 3) glucose levels 7 days after LCMV injection (Fig. 5B); 4) the time of diabetes presentation (Fig. 5C); 5) the level of hyperglycemia at presentation (Fig. 5D); or 6) the level of hyperglycemia after 3 weeks of mild insulin treatment (Fig. 5E).

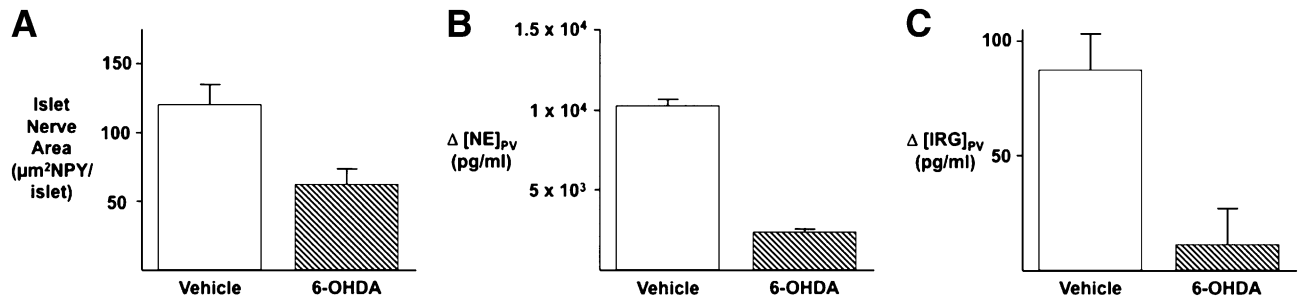


Figure 3—Loss of islet sympathetic nerves and impaired norepinephrine (NE) and glucagon (IRG) responses to tyramine in 6-OHDA-treated Ins2-GP^{Tg} mice. Quantification of islet NPY-positive nerve fibers in vehicle- and 6-OHDA-treated Ins2-GP^{Tg} mice (A). The portal venous (PV) NE response (Δ) to tyramine in vehicle- and 6-OHDA-treated Ins2-GP^{Tg} mice (B). The PV IRG response to tyramine in vehicle- and 6-OHDA-treated Ins2-GP^{Tg} mice (C).

Deleting p75NTR Prevents the Loss of Islet Sympathetic Nerves

To directly test the hypothesis that the activation of p75NTR triggers the destruction of islet sympathetic nerves in autoimmune diabetes, we looked for the preservation of these nerves in LCMV-injected, Ngfr^{-/-} Ins2-GP^{Tg} mice at the same two times when LCMV-injected Ngfr^{+/+} Ins2-GP^{Tg} mice displayed a major loss of islet sympathetic nerves.

After 3 weeks of LCMV-induced diabetes in Ngfr^{-/-} Ins2-GP^{Tg} mice, the islet sympathetic nerve area was not significantly different from that of age-matched Ngfr^{-/-} Ins2-GP^{Tg} mice not treated with LCMV (Fig. 6A, hatched bars; both *n* = 6), in marked contrast to the ~80% loss of islet sympathetic nerves seen in Ngfr^{+/+} Ins2-GP^{Tg} mice after 3 weeks of diabetes (Fig. 6A, open bars).

Likewise, 7 days after LCMV injection into Ngfr^{-/-} Ins2-GP^{Tg} mice, islet sympathetic nerve area was not

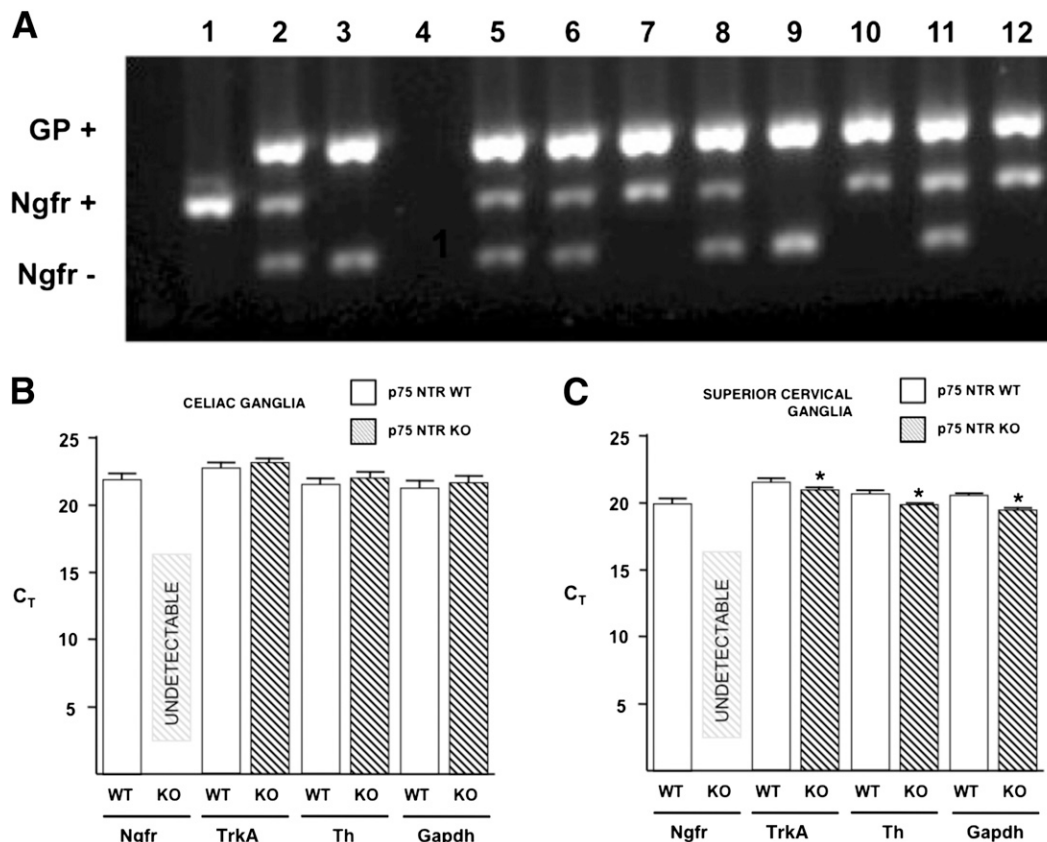


Figure 4—Deletion of Ngfr in Ins2-GP^{Tg} mice has no effect on TrkA expression in CG. A: Representative genotyping gel for the Ngfr and Ins2-GP genes in Ngfr^{+/+} Ins2-GP^{Tg} mice (lanes 7, 10, and 12), Ngfr^{+/-} Ins2-GP^{Tg} mice (lanes 2, 5, 6, 8, and 11), and Ngfr^{-/-} Ins2-GP^{Tg} mice (lanes 3 and 9). C_T values for p75NTR Ngfr, TrkA, Th, and Gapdh mRNA in CG (B) or SCG (C) in Ngfr^{+/+} Ins2-GP^{Tg} (wild type [WT], open bars) or Ngfr^{-/-} Ins2-GP^{Tg} (KO, hatched bars) mice. *Significant difference (*P* < 0.001) compared with p75NTR WT control.

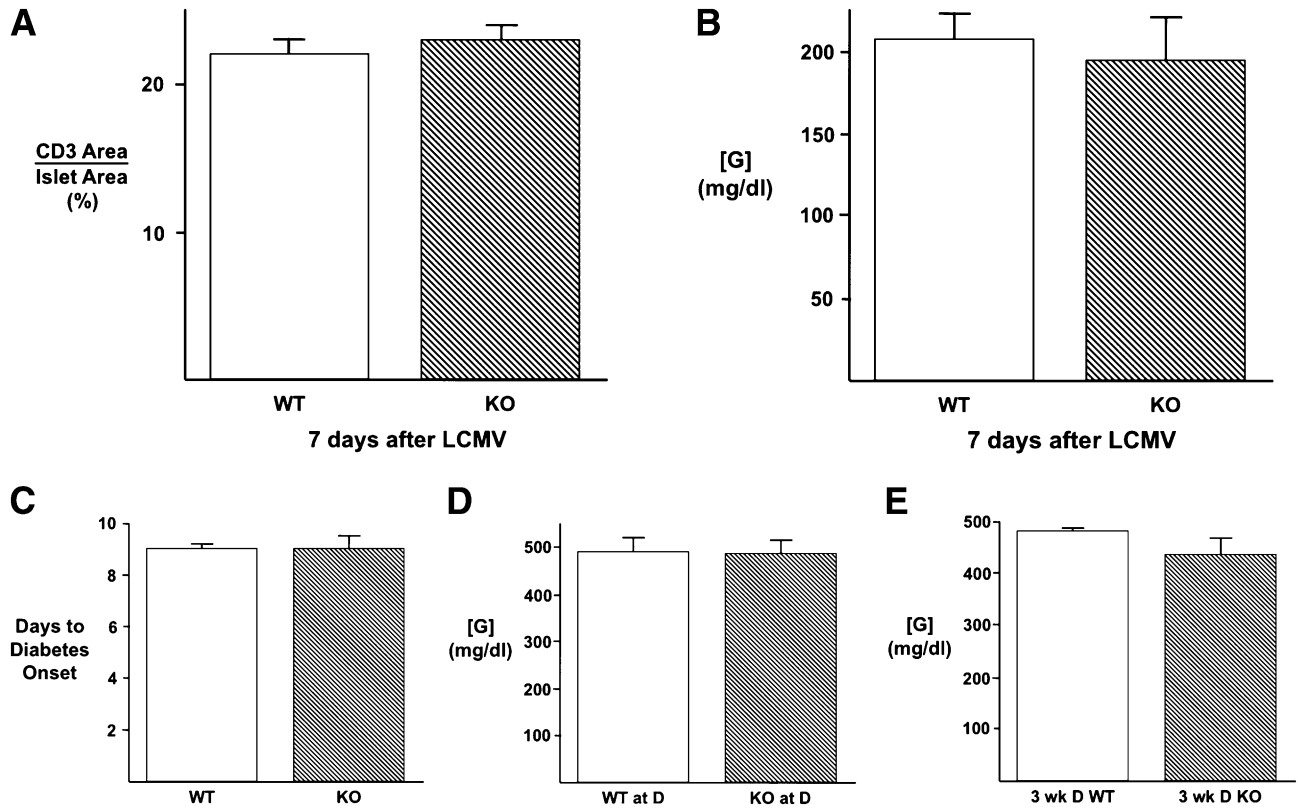


Figure 5—No effect of deleting *Ngfr* from *Ins2-GP^{Tg}* mice on T-lymphocytic infiltration, blood glucose levels, or presentation of diabetes. In all graphs, the open bar is from *Ngfr^{+/+}* *Ins2-GP^{Tg}* (wild type [WT]) mice, and the hatched bar is from *Ngfr^{-/-}* *Ins2-GP^{Tg}* (KO) mice. T-lymphocytic infiltration quantified by CD3 staining 7 days after LCMV injection (A). Blood glucose ([G]) level 7 days after LCMV injection (B). Days to presentation of diabetes after LCMV injection (C). Blood glucose ([G]) levels at diabetes presentation (at D). Blood glucose levels after 3 weeks of diabetes (3 wk D) (E).

significantly different from that of age-matched *Ngfr^{-/-}* *Ins2-GP^{Tg}* mice not injected with LCMV (Fig 6B, hatched bars; both *n* = 6), in marked contrast with the

~60% loss of islet sympathetic nerves seen in *Ngfr^{+/+}* *Ins2-GP^{Tg}* mice 7 days after LCMV injection (Fig. 6B, open bars).

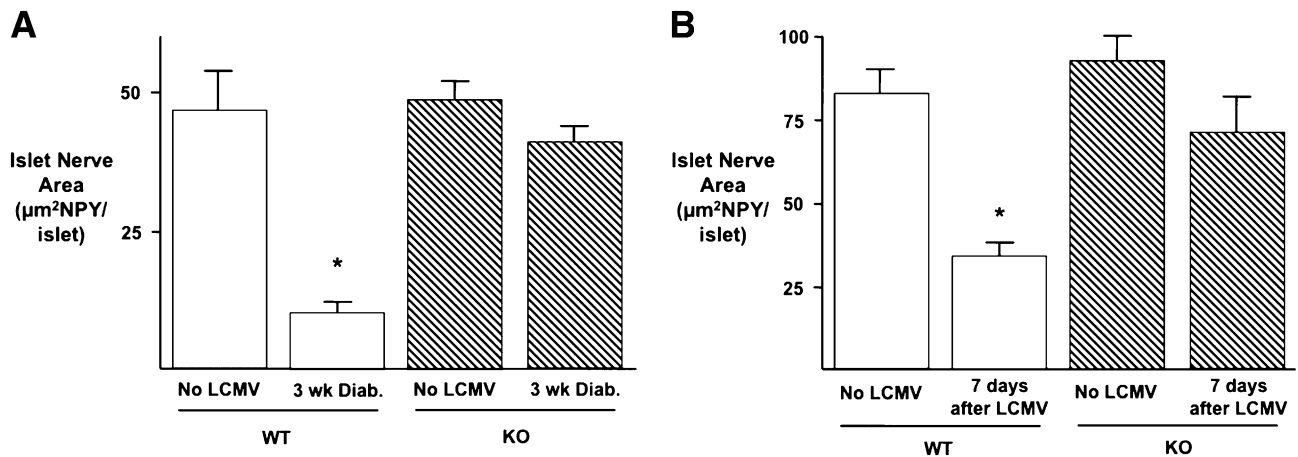


Figure 6—Deletion of *Ngfr* from *Ins2-GP^{Tg}* mice prevents the major loss of islet NPY nerves after LCMV injection or diabetes. Islet NPY-positive nerve area before or after 3 weeks of diabetes (3 wk Diab.) in *Ngfr^{+/+}* *Ins2-GP^{Tg}* mice (wild type [WT], open bars) or in *Ngfr^{-/-}* *Ins2-GP^{Tg}* mice (KO, hatched bars) (A). Islet NPY-positive nerve area before or 7 days after LCMV injection in *Ngfr^{+/+}* *Ins2-GP^{Tg}* mice (WT, open bars) or in *Ngfr^{-/-}* *Ins2-GP^{Tg}* mice (KO, hatched bars) (B). *Significant difference (*P* = 0.0002) compared with no LCMV control.

In summary, deleting the p75NTR prevents a major loss of islet sympathetic nerves both early during invasive insulinitis and after 3 weeks of diabetes. Taken together, these data demonstrate that the immune-mediated loss of islet sympathetic nerves is dependent on the p75NTR, whereas the immune-mediated loss of islet β -cells is not (see DISCUSSION).

DISCUSSION

The major finding of this study is that the p75NTR is required for the marked loss of islet sympathetic nerves that occurs when the pancreatic islet is under immune attack. The demonstration of abundant mRNA for the p75NTR in pancreas-projecting sympathetic neurons implies that direct activation of this receptor on islet sympathetic axons is the cause of this rapid nerve loss. Our findings are a major step forward in defining the mechanism by which islet sympathetic nerves are destroyed early in immune-mediated diabetes.

The current study was also able to rule out four alternative mechanisms for the loss of islet sympathetic nerves. First, viral infection did not cause this eSIN because there was no loss of sympathetic nerves 4 days after LCMV injection, when systemic virus is present in this model (29). Second, since deleting the p75NTR did not prevent or alter the development of diabetes, it is unlikely that the T lymphocytes that directly attack islet β -cells also directly attack islet sympathetic nerves. Third and fourth, neither β -cell loss nor diabetic hyperglycemia is a cause of eSIN since p75NTR KO mice with 3 weeks of LCMV-induced diabetes have normal islet sympathetic innervation. In summary, eSIN is not caused by direct viral infection, β -cell loss, or diabetic hyperglycemia, but rather it is triggered indirectly when the lymphocytes invade the islet.

The severity of the nerve loss that occurs in this inducible Tg model of immune-mediated diabetes is similar to that seen in naturally occurring autoimmune diabetes. For instance, in Ins2-GP^{Tg} mice, the loss of islet sympathetic nerves, assessed by either NPY or Th staining (Supplementary Fig. 2), reaches 80% after 3 weeks of diabetes, compared with 66% as assessed by NPY staining in diabetic NOD mice (21), 85% as assessed by vesicular monoamine transporter 2 staining in diabetic BB rats (20), and 93% as assessed by Th staining in type 1 diabetic human pancreas (23). Thus, each model of immune-mediated diabetes showed marked nerve loss, independent of the nerve marker used.

These degrees of nerve loss are sufficient to markedly impair the glucagon response to sympathetic activation, as we previously demonstrated directly in diabetic BB rats (24) and diabetic NOD mice (21). Such direct demonstration was not possible in the Ins2-GP^{Tg} mouse since the LCMV injection renders these mice a BSL2 class biohazard. However, a chemically induced nerve loss, similar to that induced by LCMV injection, markedly impaired the glucagon response to their activation.

The relevance of this finding to humans could be questioned since in humans, sympathetic nerves innervate primarily the islet vasculature (30), whereas in mice, they also directly contact islet endocrine cells (11,30). Surprisingly, an infusion of tyramine in humans produced the inhibition of insulin release expected during activation of islet sympathetic nerves (31), perhaps because they release their neurotransmitter directly into the arteriole perfusing the islet. Thus, although the anatomic arrangement of islet innervation differs between mice and men, islet sympathetic nerves still influence hormone secretion in humans.

The islet selectivity of the nerve loss that occurs in LCMV-injected Ins2-GP^{Tg} mice also occurs in diabetic BB rats (20) and in patients with type 1 diabetes (23). Thus, despite differences in the speed of onset and severity of autoimmune diabetes between type 1 diabetic humans and animal models thereof, once marked lymphocytic infiltration has occurred, as judged by CD3 staining in mice and by marked β -cell loss in humans, the severity of the nerve loss and its islet selectivity are similar. Thus, the mechanism described in this study for the loss of islet sympathetic nerves in Ins2-GP^{Tg} mice is a strong candidate for causing the loss of these nerves in human type 1 diabetes.

This mechanism clearly involves the p75NTR since its global KO prevents the majority of islet nerve loss. Before we could ascribe this loss specifically to the p75NTR that resides on sympathetic axons, as has been done for the pruning of excess sympathetic axons during the development (26), other possible effects of our global KO first had to be ruled out.

One possibility is that knocking out the p75NTR might increase basal sympathetic innervation, thereby simply offsetting the nerve loss induced by the immune attack of the islet. Although there is increased sympathetic innervation of the submandibular gland in p75NTR KO mice (32), consistent with our finding of increased Th and TrkA expression in the SCG that supplies the sympathetic innervation to this tissue, we found no such effect on the basal sympathetic innervation of the islet and no increase of Th or TrkA expression in the celiac ganglia from which islet innervation comes (28). Another possibility is that knocking out the p75NTR might decrease T-lymphocytic infiltration and thereby prevent the loss of islet sympathetic nerves since, in the NOD mouse, eSIN can be prevented by blocking invasive insulinitis (21). However, infiltration of T lymphocytes, assessed by CD3 staining in LCMV-treated Ins2-GP^{Tg} mice, was not affected by knocking out the p75NTR. The third possibility is that knocking out the p75NTR spares islet sympathetic nerves because the neurotrophin receptor (TrkA), which mediates the beneficial effect of nerve growth factor (NGF) on sympathetic nerves (33,34), is upregulated. While our mRNA data suggest that this may be true for the SCG, it is unlikely to be true for the CG, from which sympathetic axons project to

the pancreas (28). Thus, it is unlikely that the rescue of islet sympathetic nerves is due to an increase of the neuroprotective receptor on their axons.

Given that the expression of *Ngfr* and *TrkA* are similar in CG, increased islet expression of an agonist that is selective for p75NTR could cause eSIN. Brain-derived neurotrophic factor (BDNF) is such a selective agonist since it binds to p75NTR but not to *TrkA* (35). Further, increased BDNF is known to cause degeneration of excess sympathetic axons, at least during development (26), especially if NGF is deficient (26). Islet NGF may be deficient in our model since β -cells are a major source of islet NGF (36), and they are under immune attack. Demonstrating an increase of islet BDNF is likely to be difficult and may require using Tg mice, which coexpress an antigenic tag with BDNF. These models have proved useful in studying the role of BDNF in other tissues (37,38).

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