BDNF/TrkB signaling in pancreatic islet beta cells

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a secreted hormone present in the blood that is an essential participant in whole body energy homeostasis. The best characterized actions of BDNF and its receptor tyrosine kinase, TrkB, are in synaptic plasticity, learning and memory in the nervous system. TrkB is also expressed in multiple other tissues including human and rodent pancreatic islets. Because little is known about BDNF signaling in pancreatic islet cell types, we examined activation of ERK1/2 by BDNF and interactions with regulators of insulin secretion. Both MIN6 β cells and αTC1-9 α cells responded to physiological BDNF concentrations. ERK1/2 were no longer activated by BDNF in TrkB-deleted cells; signaling to ERK1/2 was restored in these cells by re-expression of full-length TrkB, confirming that TrkB is required for BDNF signaling. BDNF potentiated activation of ERK1/2 by glucose and GLP-1 in MIN6 cells without detectably altering cAMP. Activation of ERK1/2 by BDNF was independent of calcium influx but required Raf1; however ERK activation by BDNF was blocked by epinephrine, an inhibitor of insulin secretion, in a Gi/o-dependent manner. Surprisingly, epinephrine also blocked BDNF-induced tyrosine phosphorylation of TrkB. We conclude that BDNF acting through its receptor TrkB has previously unrecognized functions in endocrine cells of the pancreas.

BDNF and its receptor tyrosine kinase the neurotrophic receptor tyrosine kinase 2 (TrkB) mediate aspects of neuronal development and differentiation and are essential in whole body energy homeostasis (1,2). They are also implicated in cancer (3-5) and diabetes (6). Highly expressed in the nervous system, TrkB has more recently been found in chondrocytes (1,2), adipose tissue (7), cardiac and skeletal muscle (8,9), liver (10), and oocytes (11), for example, implying a wide range of functions throughout the body.

BDNF can regulate islet hormones, like glucagon, through actions in the hypothalamus and innervation of the islet (12) and is suggested to have direct effects on alpha cells (13). TrkB is expressed in multiple islet cell types including rodent and human β and α cells (13-17), and usually to a greater extent than the related receptor tyrosine kinase TrkA. TrkA and its ligand nerve growth factor (NGF) function in β cell survival, pancreatic innervation, and are regulated by glucose (18-24).

In spite of evidence for greater expression of BDNF-TrkB in islet cell types, little is known about their actions and signaling mechanisms in the islet. There are multiple studies linking BDNF levels to type 2 diabetes (T2D) in humans and mice (25-28) as well as in type 1 diabetic patients (29). Among these studies are numerous lines of evidence connecting BDNF to glucose

homeostasis and which implicate islets as targets. More than 15 years ago, BDNF was found to regulate glucose metabolism in mice (30). $BDNF^{+/-}$ mice became obese and had elevated serum leptin and insulin, but maintained normoglycemia (31). BDNF null animals usually die by the second post-natal week and the newborn knockout mice have elevated blood glucose (13). Treating db/db mice with BDNF lowered blood glucose and increased pancreatic insulin content (32). β cell area and staining intensity were increased and pancreatic glucagon content was decreased (33). BDNF may also have a cytoprotective role in the islet because treatment with BDNF prevented RIN5F β cell death in response to alloxan, streptozotocin, doxorubicin and benzo(a)pyrene (34). These studies indicate the need for futher analysis of the effects of BDNF-TrkB signaling in pancreatic islet β cells.

In view of these findings on BDNF and glucose homeostasis, we examined the impacts of BDNF signaling on cell function and ERK1/2 activation in β cell lines. ERK1/2 regulation by secretagogues mimics regulation of insulin secretion and is frequently used as a proxy for secretion (35-37). Furthermore, ERK2 activity has recently been shown to be critical for first-phase insulin secretion (38). Our analysis of ERK1/2 activation revealed unexpected interactions of BDNF with insulin secretagogues and secretion inhibitors.

Results

TrkB in pancreatic β cells promotes insulin secretion and cell growth

We probed human and mouse islets to determine if TrkB protein is present in β and α cells. Using insulin and glucagon as markers, we detected expression of TrkB in both cell types in normal human (**Fig. 1A**) and mouse (**Fig. 1B**) pancreatic islets by immunocytochemistry with multiple antibodies. Testing of these antibodies on cells that overexpressed TrkB indicated that the Millipore antibody most accurately represented receptor expression (**Fig. S1A**). Because TrkB expression was detected in multiple islet cell types, we used MIN6 cells to specifically test the impact of BDNF signaling on β cells. We observed that chronic 24 h treatment of MIN6 cells with BDNF resulted in an increased fold response of insulin secretion after 30 min of glucose stimulation (**Fig. 1C**). 48 h of BDNF treatment caused an increase in a Cell Titer Blue viability assay readout (**Fig. 1D**), potentially a result of the mitogenic qualities of BDNF.

BDNF activates the ERK1/2 MAP kinase pathway in β cells

Similar to other TrkB-expressing cell types, we found that clonal rodent β cell lines respond to BDNF leading to activation of ERK1/2 within 5 minutes of stimulation (**Fig. 2A, S1B**). We found an increase in pERK1/2 with as little as 2.5 ng/ml; and activation plateaued at ~10-20 ng/ml (**Fig. 2A, S1C**), well within the range of the circulating hormone. BDNF concentrations in human blood are reported ranging from of 5 to 2900 ng/ml in plasma (25,39-41), while serum concentrations can be much higher (9,42). BDNF had a negligible effect on the phosphorylation of Akt, but increased S6 phosphorylation at 30 min, suggesting activation of the mTORC1 pathway (**Fig. 2B**). We also observed that chronic stimulation with low or high doses of BDNF in MIN6 cells led to a prolonged ERK1/2 activation (**Fig. S1D**). Additionally, we tested synthetic ligands that had been suggested to activate TrkB but we were unable to demonstrate any effects in MIN6 cells (not shown), consistent with recent findings concerning the inactivity of these ligands (43).

Previously we found that activation of ERK1/2 by glucose required B-Raf acutely and Raf1 (aka C-Raf) for long term stimulation (44). Raf1 has been shown to be required for insulin receptor signaling and insulin expression and content in β cells (45). To determine the Raf isoform(s) used by BDNF to signal to ERK1/2 in MIN6 cells, we generated CRISPR/Cas9-based Raf1 knockouts. In four MIN6 β cell knockout lines that were recovered, we found that loss of Raf1 prevented BDNF signaling to ERK1/2 (**Fig. 2C**). In contrast, loss of Raf1 had a much less dramatic effect on EGF signaling to ERK1/2 which was still readily detectable. These results suggest that BDNF and EGF differentially utilize MAPK cascade components to activate the

pathway in β cells. We did not measure differences in B-Raf use, because the B-Raf knockout clones we obtained from MIN6 cells failed to survive.

TrkB is required for BDNF signaling in MIN6 β cells

Blockade of TrkB signaling by the TrkB-selective inhibitor GNF-5837, as well as the pan-Trk inhibitor lestaurtinib, prevented BDNF-induced ERK1/2 activation in MIN6 cells (**Fig. 2D, S1E**). To complement these pharmacological data and ensure that we were not observing signaling through TrkA, we also took a genetic approach. We used CRISPR/Cas9 gene editing to knock out TrkB from MIN6 β (**Fig. 2E**). We isolated multiple clonal lines of MIN6 cells from which TrkB had successfully been disrupted, confirmed by immunoblotting. The knockout cells were verified to retain glucose-induced ERK1/2 activation (**Fig. S1F**). The TrkB knockout lines no longer responded to BDNF, confirming that TrkB is the major receptor required for BDNF-induced ERK1/2 activation in MIN6 cells (**Fig. 2E**, pIRES lanes). Multiple isoforms of TrkB are expressed from the NTRK2 gene, with the major forms being full-length kinase domain-containing TrkB (TrkB-FL) and a truncated form, TrkB-T1, which is missing the kinase domain (46). To validate that TrkB-KO MIN6 cells retained the downstream TrkB signaling components, we tested whether the expression of TrkB by transient transfection of TrkB-FL, TrkB-T1, or both together could rescue BDNF-stimulated ERK1/2 activation. As expected, only re-expression of TrkB-FL in the knockout cells restored ERK1/2 activation in response to BDNF stimulation (**Fig. 2E**), whereas TrkB-T1 lacking the kinase domain did not.

BDNF enhances glucose- and GLP-1-induced ERK1/2 activation and is ablated by epinephrine

Circulating BDNF concentrations are altered in diabetes and BDNF acts through multiple pathways present in β cells. Therefore, we asked whether BDNF exhibits crosstalk with β cell pathways that regulate insulin secretion. We tested the ability of BDNF to interact with pro-secretory (GLP-1/GLP-1R) and inhibitory (epinephrine/α_2-adrenergic receptor) signaling pathways, as well as glucose alone (**Fig. 3A**). BDNF activated ERK1/2 in the absence of glucose and enhanced their activation in the presence of different concentrations of glucose (**Fig. S1G**). We found that, whereas BDNF and glucose each activate ERK1/2 on their own, in combination they synergize to yield more than additive ERK1/2 activity (**Fig. 3B**, control). Cross-talk between receptor tyrosine kinases and GPCRs has been found repeatedly (47), although little is known about the relevance of such pathway interactions in islets. Pre-treatment with GLP-1 enhanced the ERK1/2 response to the combination of BDNF and glucose (**Fig. 3B**), suggesting interactions among TrkB, the GLP-1 receptor, and glucose metabolic pathways. We hypothesized that BDNF acts in tandem with other metabolic hormones to regulate signaling in the β cell. Interestingly, epinephrine treatment dramatically inhibited ERK1/2 activation in response to either glucose, BDNF or their combination (**Fig. 3B**).

Epinephrine blocks BDNF-induced TrkB signaling through $G_{i/o}$ in β cells

Epinephrine has different effects on the ERK1/2 MAPK pathway depending on cell type and receptors expressed. In β cells, epinephrine activates $\alpha 2_{A/C}$-adrenergic receptors and inhibits insulin secretion as well as glucose-stimulated ERK1/2 activation (48,49). Previously we found that NGF (which signals through TrkA) activates ERK1/2 normally in the presence of epinephrine (49). This is in contrast to our observation that pretreatment of MIN6 cells with epinephrine prevented BDNF-stimulated ERK1/2 activation (**Fig. 3B**). Inhibition likely occurs through the α_2-adrenergic receptor because the α_2-adrenergic-selective agonist UK14304 also inhibited ERK1/2 activation by BDNF (**Fig. S1H**). Unexpectedly, we found that BDNF-induced TrkB tyrosine phosphorylation was strongly suppressed by epinephrine (**Fig. 3C**), suggesting potentially direct effects on the TrkB receptor tyrosine kinase.

Epinephrine inhibits adenylyl cyclase and insulin secretion in β cells through α_2-adrenergic receptors linked to $G\alpha_{i/o}$. To delve further into the mechanism of epinephrine-mediated blockade of BDNF-TrkB signaling, we treated cells with

pertussis toxin (PTX) which inactivates $G\alpha_{i/o}$. INS1 β cells were used in this case because they responded well to BDNF stimulation and exhibited a more robust response to PTX than MIN6 cells. PTX rescued the effects of epinephrine on BDNF- and glucose-mediated activation of ERK1/2 (**Fig. 4A**).

BDNF action is independent of calcium and cAMP signaling in MIN6 β cells

We examined second messengers, calcium influx and cAMP generation, which are both known to regulate ERK1/2 in β cells and be inhibited by epinephrine/$G_{i/o}$. Prevention of calcium influx was achieved by removal of calcium from the incubation buffer. Without extracellular calcium, BDNF signaling to ERK1/2 remained intact while KCl-mediated depolarization no longer activated ERK1/2 (**Fig. 4B**), demonstrating a dichotomy between growth factor and depolarization-stimulated ERK activation. Additionally, inhibition of BDNF-induced ERK activity by epinephrine was independent of calcium influx (**Fig. 4B**).

We tested whether BDNF can induce cAMP generation in MIN6 cells expressing a bioluminescence-resonance energy transfer-based cAMP reporter (cAMP sensor using YFP-Epac-RLuc or CAMYEL) (50,51). While the known G_s activator GLP-1 increased cAMP (52), BDNF had no effect on cAMP, either alone or in combination with glucose or GLP-1 (**Fig. 4C**). We also tested inhibitors of adenylyl cyclase (dideoxyadenosine and SQ22536) and found no impact on BDNF signaling (**Fig. S1I**).

A previous report suggested that inhibition of PI3K or PDK1 activity abrogates the hyperpolarizing effects of micromolar epinephrine treatment (53). We pre-treated MIN6 cells with well-characterized PI3K and PDK1 inhibitors followed by epinephrine treatment and analyzed the response to BDNF. These inhibitors did not rescue BDNF-induced ERK1/2 activation (**Fig S2**), suggesting the PI3K/PDK1 pathway is not required for the G_i-dependent blockade of TrkB activation.

It is possible that epinephrine inhibits other receptor tyrosine kinase signaling cascades in addition to BDNF/TrkB. To test this, we analyzed its effects on EGF signaling. We found EGF signaling to ERK1/2 was partially inhibited with epinephrine pre-treatment (**Fig 4D**), however BDNF-TrkB signaling appeared more sensitive. Recent findings suggest supraphysiologic (μM) doses of epinephrine inhibit insulin secretion by hyperpolarizing the plasma membrane, while more physiologic (nM) doses block cAMP/TRPM2 activation without substantial hyperpolarization (54). We treated MIN6 cells with 5 nM or 5 μM epinephrine and tested the response to BDNF and EGF. 5 nM epinephrine was unable to suppress either BDNF or EGF signaling to ERK, nor TrkB tyrosine phosphorylation, and while 5 μM epinephrine blocked BDNF signaling, EGF retained significant ability to activate ERK1/2 (**Fig 4D**).

Discussion

Our results suggest that physiological concentrations of BDNF impact signal transduction through TrkB in β cells and likely other islet cell types. NGF and its receptor TrkA have been linked to β cells for a number of years (18,20,21,49,55). A recent study concluded that NGF and TrkA have a critical role in β cell function in mice and in isolated islets from mice and humans (21). In that study, blockade of NGF-TrkA signaling in mice using chemical genetics and pancreas-specific TrkA knockout impaired insulin secretion and glucose tolerance. Glucose also induced TrkA phosphorylation (21). In several studies of NGF action on islets including those discussed here and our previous work (49), pharmacological concentrations of NGF were used, up to 50 times higher than the concentration of BDNF needed to induce ERK1/2 activation. Yet, NGF is present in the circulation at lower concentrations than BDNF (0.01-2 ng/ml NGF in humans) (56-60). NGF signaling to islets *in vivo* may be due to local concentrations around the islet which may be higher than in the circulation. Because the phosphorylated Trk antibodies that are available cannot distinguish between the phosphorylated TrkA and TrkB, it is possible that data collected using these antibodies represent both TrkA and TrkB phosphorylation, or that TrkB exhibits crosstalk with TrkA (61). TrkB is known to exhibit crosstalk with other kinases, including

Src-family kinases (62), Ret (63) and the EGF receptor (64). Oligomerization between receptor kinases TrkA and TrkB may be a significant contributor to the actions of NGF and may also contribute to BDNF-TrkB signaling not reflected by ERK1/2 activity. Another factor that may complicate interpretation of BDNF function is the expression of isoforms lacking the kinase domain. In our rescue experiments TrkB-T1 seemed to impair the activity of the full length receptor, as has been suggested in the literature (65-67). Therefore, it remains to be determined in the whole animal whether specific deletion of TrkB from different islet cell types during development or inducibly in the adult will uncover or deconvolve roles for TrkB vs TrkA in islet development and function.

ERK1/2 activation by glucose is dependent on multiple factors such as calcium influx (68), the calcium-dependent phosphatase calcineurin (44), and Cdc42/PAK1 activation (69,70). BDNF signaling appears to be independent of at least the first two of these glucose requirements. Calcineurin dephosphorylates a site of ERK negative feedback phosphorylation on B-Raf (44). This negative feedback is reversed by calcineurin so that B-Raf can respond to glucose and other secretagogues to activate the ERK1/2 cascade. Because its actions are calcium independent, BDNF must have a different mechanism to overcome negative feedback on B-Raf. B-Raf and Raf1 can both lead to ERK1/2 activation. By depending on Raf1, BDNF apparently bypasses pathway blockade due to B-Raf negative feedback. In the future, determining whether BDNF also signals through B-Raf will likely require its conditional deletion.

In addition to the events that distinguish signaling by BDNF and glucose, the unexpected sensitivity of BDNF to inhibition by α_2-adrenergic agonists also suggests a connection between signaling by BDNF and insulin secretagogues. In general, receptor tyrosine kinases share certain signaling pathways; the Ras-ERK1/2, PI3K-Akt and PLCγ pathways are the most recognized (71). Depending on context, different ligand-receptor family members may signal independently within the same cell to different pathways or exhibit inter-pathway crosstalk (72), but the exact molecular mechanisms are unclear. Additionally, other inputs such as glucose-stimulated metabolic pathways can act on some of the same signaling pathways seemingly by independent mechanisms. (70,73,74). α_2-adrenergic signaling is well-known to antagonize insulin secretion in β cells, largely through heterotrimeric $G_{i/o}$ proteins (49,54,75,76). In tandem with this effect, α_2-adrenergic signaling blocks glucose stimulated ERK phosphorylation (49), presumably due to membrane hyperpolarization, inhibition of adenylyl cyclase and blockade of calcium influx. We found that BDNF signaling through the Raf-MEK-ERK pathway is also blocked in β cells by epinephrine through a $G_{i/o}$-dependent mechanism directly acting on TrkB activation. This indicates that BDNF-TrkB and glucose-stimulated signaling pathways share certain essential regulatory inputs in the β cell.

α_2-adrenergic activation inhibiting BDNF-TrkB signaling in β cells represents an unusual example of pathway crosstalk that is dependent on $G_{i/o}$. G proteins often activate tyrosine kinase pathways or tyrosine kinases activate G proteins, but reports of G protein inhibition of receptor kinases are uncommon (47). Endogenous plasma epinephrine concentrations in humans are usually 10-100 pg/ml (77,78), but can increase to 1000 pg/ml (79) during infusions of drugs or epinephrine itself (10 pg/ml = 54.5 pM, 1000 pg/ml = 5.4 nM). Both high (μM) and low (nM) doses of epinephrine have been proposed to inhibit insulin secretion through different mechanisms (54). In the case of nanomolar concentrations of epinephrine, cAMP-TRPM2 channel activity is suppressed, blunting glucose-induced insulin secretion, although sulfonylurea-induced secretion was unaffected. At ≥1 μM epinephrine, secretion under nearly all conditions is inhibited and the membrane is hyperpolarized. In work from Peterhoff, et al. (48), 1 μM epinephrine was shown to inhibit adenylyl cyclase and hyperpolarize the plasma membrane in wild-type β cells, but not in α_{2A}-adrenergic receptor knockout β cells, suggesting the actions of micromolar concentrations of epinephrine are not occurring nonspecifically.

Because BDNF signaling in MIN6 cells appears unaffected by changes in intracellular calcium and does not induce cAMP on its own, suppression of these mechanisms are unlikely to account for the effect of epinephrine on BDNF signaling. However, we found that micromolar concentrations of epinephrine were required for its activity against BDNF-TrkB signaling. Why does nanomolar epinephrine fail to block BDNF-TrkB signaling when it is likely activating $G_{i/o}$ under those conditions? This suggests a potential mechanism dependent on membrane hyperpolarization, or perhaps through activation of G protein-gated inward rectifier potassium channels (GIRKs) (80), although it is unclear how this would intersect with TrkB. While currently untested, it is formally possible that $G_{i/o}$ binds directly to TrkB to inhibit its activation as was shown for $G_{i/o}$ binding to insulin receptor in β cells (81).

In the future it will be important to place these actions of BDNF in an *in vivo* context to assess their metabolic impact. It will also be important to elucidate the mechanism underlying the unanticipated finding that epinephrine prevents activation of TrkB by BDNF. If any components of that mechanism are pharmacologically targetable, it may be possible to modulate TrkB or other RTK signaling in islets *in vivo* for therapeutic benefit. Additionally, our findings have wide-ranging implications for other systems in which adrenergic and receptor tyrosine kinase signaling may converge, such as cancer (82-84).

Experimental Procedures

Antibodies, Plasmids, and Reagents – All chemicals were purchased through Fisher Scientific unless otherwise indicated. BDNF was from Millipore (GF029). Epinephrine (E4375) and GLP-1 (G8147) were from Sigma. Cell Titer Blue was from Promega. Antibodies, their dilutions for immunoblotting and/or immunofluorescence assays, and their source information are contained in **Table S1**. Given the many available TrkB antibodies and differences in specificity against human versus rodent, we tested five different antibodies against human SH-SY5Y cells (ATCC) treated with or without retinoic acid to induce TrkB expression (85) by immunoblotting. We determined that rabbit anti-TrkB from Abcam (Cat# ab134155, 1:1000), rabbit anti-TrkB from Cell Signaling (Cat# 4603, 1:1000), rabbit anti-TrkB from Millipore (Cat# 07-225, 1:1000) and detected full-length human TrkB. Mouse anti-TrkB from R&D (Cat# MAB397, 1:500) and mouse anti-TrkB from Santa Cruz (Cat# sc-377218, 1:500) were not optimal.

Plasmid generation – Mouse TrkB-FL and TrkB-T1 cDNAs were cloned from MIN6 cell libraries. cDNA libraries were generated using the Protoscript II First Strand cDNA Synthesis kit (NEB Cat# E6560S). Mouse TrkB-FL and TrkB-T1 cDNAs were inserted into pcDNA3.1+ and pIRES-3xFlag-dsRed using standard molecular biology techniques. pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid #42230) (86). Guide RNAs were designed targeting mouse NTRK2 exon 1 and cloned into pX330 following the Zhang lab protocol. The 20 bp guide sequence (#1: 5'-GACCCGCCATGGCGCGGCTC-3', #2: 5'-GGAACCTAACAGCGTTGACC-3') was generated using the CRISPR design tool (http://crispr.mit.edu). For cloning the guide RNA into the BbsI site of pX330, DNA oligonucleotides were synthesized: #1: sense 5'-CACCGACCCGCCATGGCGCGGCTC-3' and antisense 5'-AAACGAGCCGCGCCATGGCGGGTC-3', #2: sense 5'-CACCGGAACCTAACAGCGTTGACC-3', anti-sense: 5'-AAACGGTCAACGCTGTTAGGTTCC-3'. The oligonucleotides were phosphorylated with T4 PNK and annealed for insertion into pX330 (pX330-mNTRK2). All plasmids were verified by DNA sequencing (Genewiz).

Immunoblotting – 40-50 μg of cleared cell lysates were separated on 10% gels by SDS-PAGE and transferred to nitrocellulose for immunoblotting. All membranes were blocked in Odyssey blocking buffer (Licor) for 1 h before overnight incubation with primary antibodies diluted in blocking buffer. After three 10 min washes in 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween-20 (TBS-T), membranes were incubated with fluorescent secondary antibodies for 1 h at room temperature.

After three 10 min washes in TBS-T, membranes were imaged on a Licor Odyssey scanner.

Cell culture and transfections – MIN6 β cells were cultured in Dulbecco's modified Eagle's medium (which contains 25 mM glucose), supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine, and 50 μM β-mercaptoethanol as described previously (87). MIN6 cells in 12 well dishes were untreated or transfected with Lipofectamine 2000 (for plasmids) (Life Technologies, Inc) according to the manufacturer's instructions and cultured 48 h before use in experiments. For BDNF chronic treatments, cells were incubated with 100 ng/ml BDNF in complete culture media as indicated in the figure legends. Prior to stimulation, MIN6 cells were washed twice with and incubated for 2 h in freshly prepared glucose-free modified Krebs-Ringer bicarbonate buffer (MKRBB: 5 mM KCl, 120 mM NaCl, 15 mM HEPES, pH 7.4, 24 mM NaHCO3, 1 mM MgCl2, 2 mM CaCl2, and 1 mg/ml radioimmunoassay-grade BSA). For insulin secretion measurements, supernatents were collected, cleared and stored at -20°C. Cells were lysed in 25 mM HEPES, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM NaCl, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml leupeptin and cleared of insoluble material by centrifugation at 10,000 x g for 10 min at 4°C for subsequent use. Secreted and total insulin content were measured using a mouse Insulin ELISA (Mercodia).

To generate MIN6 TrkB knockout lines, 35 mm wells of MIN6 cells were co-transfected with 2.5 μg of pX330 containing gRNAs and 1μg of pEGFP-C2 (Clontech). After 48 h, cells were trypsinized and prepared for fluorescence activated cell sorting. GFP positive cells were sorted one per well in round bottom 96-well trays (Corning) for clonal expansion. After 1-2 weeks, colonies were observable in 10-20 wells per tray. After 1-2 more weeks of growth, colonies were trypsinized and seeded into 24-well plates and screened for TrkB protein expression by immunoblotting. Clones which exhibited normal morphology and low basal phosphorylated ERK1/2 (pERK1/2) were chosen for studies. The parental MIN6 line was used as a wild-type control.

Rat INS-1 cells were cultured in RPMI-1640 containing 11 mM glucose, 10% FBS, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 10 mM Hepes pH 7.4, penicillin/streptomycin as above. Conditions for pertussis toxin (PTX, Fisher PHZ1174) treatments have been described previously (49,88). Briefly, INS1 cells in 12-well culture dishes were incubated for 24 h in the presence or absence of 200 ng/ml PTX, followed by a 2 h incubation in KRBH with 2 mM glucose also in the presence or absence of 200 ng/ml PTX. The cells were then pretreated with 10 μM epinephrine for 15 min and stimulated with 10 ng/ml BDNF or 20 mM glucose for the indicated times and harvested for immunoblotting.

Intracellular cAMP measurements – We used the Epac-based bioluminescence resonance energy transfer (BRET) sensor for cAMP (CAMYEL) in MIN6 cells as previously described (50,51). Briefly, BRET assays were performed on the Synergy H1 microplate reader (BioTek). MIN6 CAMYEL cells were preincubated in KRBH without glucose for 1.5 h followed by addition of 2.55 μM coelenterazine-h for 15 min. Emission signals at 485/20 and 528/20 nm (center/bandpass) were measured every 0.8 s during a 3 min baseline measurement. Stimuli were then added cells were measured for an additional 10 min.

Microscopy – Paraffin-embedded formalin-fixed sections of de-identified human pancreas tissue on glass slides were obtained through the Simmons Comprehensive Cancer Center at UT Southwestern Medical. Slides were deparaffinized with the assistance of the UTSW Molecular Pathology Core using an automated system for xylene and ethanol washes. Antigen retrieval was performed by heating in citrate buffer as described (http://www.ihcworld.com/_protocols/epitope_retrieval/citrate_buffer.htm). After three 10 min washes in PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 0.05% Tween-20), slides were blocked for 1 h at room temperature in normal donkey serum (NDS) block solution (2% donkey serum, 1% bovine serum albumin, 0.1% cold fish skin gelatin, 0.1% Triton

X-100, 0.05% sodium azide, PBS-T). Sections were outlined with a barrier pen and incubated overnight at 4°C with primary antibodies. Primary antibodies were diluted in NDS blocking solution at the indicated dilutions (**Table S1**). After three 10-min washes in PBS-T, slides were incubated in secondary antibodies in NDS block for 1 h at room temperature. The washed slides were mounted with Dapi Fluoromount-G (SouthernBiotech #0100-20) and imaged on either an LSM700 or LSM780 Zeiss confocal microscope.

Statistical Analysis – Quantitated data are expressed as mean ± SE. Data were evaluated using Student's t test, or ANOVA with appropriate multiple comparisons test where indicated, and considered significant if $P < 0.05$. Graphs were made in GraphPad Prism 7.

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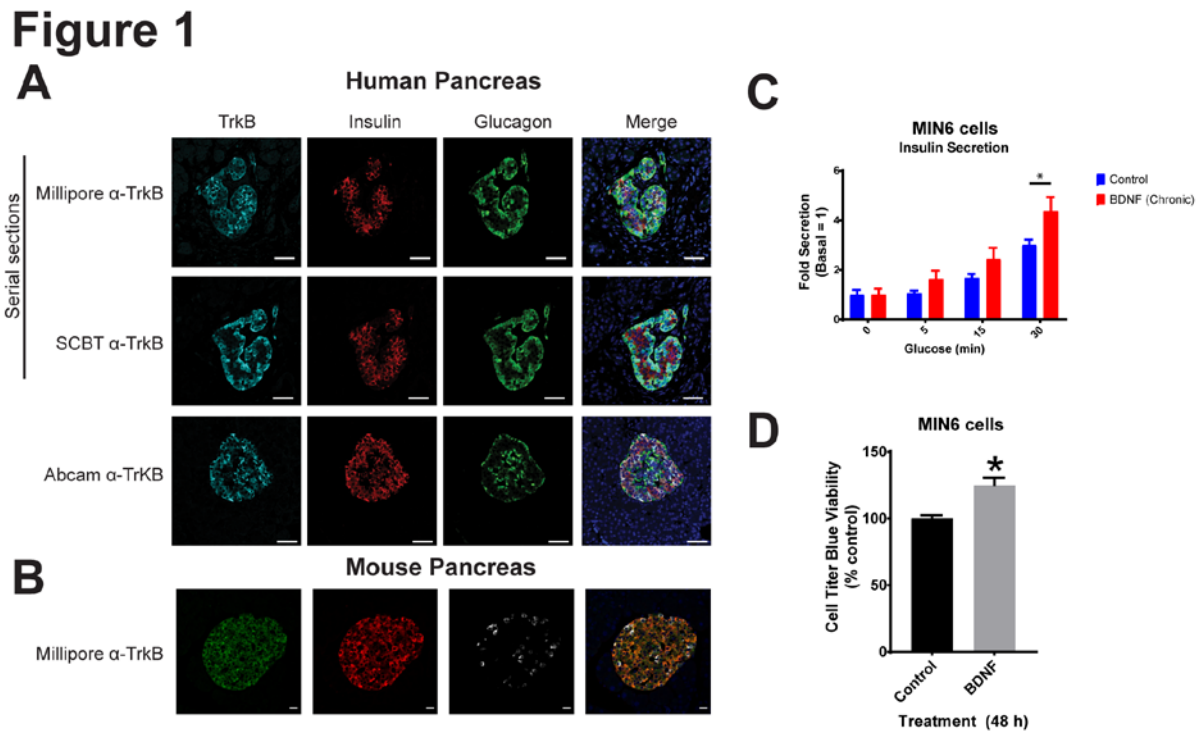

Figure 1. TrkB is expressed in pancreatic islets and chronic BDNF signaling promotes insulin secretion and β cell growth. A) Human pancreas tissue sections were immunostained with antibodies against TrkB, insulin, glucagon and also stained with DAPI. For Millipore and Santa Cruz anti-TrkB antibodies, serial sections from the same tissue block were stained with different TrkB antibodies. Data are representative of at least 3 images from 2 human pancreas tissue donors. Scale bar, 50 μm. **B)** Wild-type mouse pancreas tissue sections were immunostained for TrkB, insulin, and glucagon. Scale bar, 20 μm. **C)** MIN6 cells were incubated with BDNF (100 ng/ml) for 24 h followed by incubation in KRBH to assay for glucose-stimulated insulin secretion. Insulin secretion (as % content) was normalized to basal secretion. Bar graph is the mean ± SE from three independent passages of cells. *, P<0.05. **D)** MIN6 cells plated in 96 well dishes were incubated for 48 h with BDNF (100 ng/ml) followed by Cell Titer Blue assay for viability. Bar graph is the mean ± SE from three independent passages of cells. *, P<0.05.

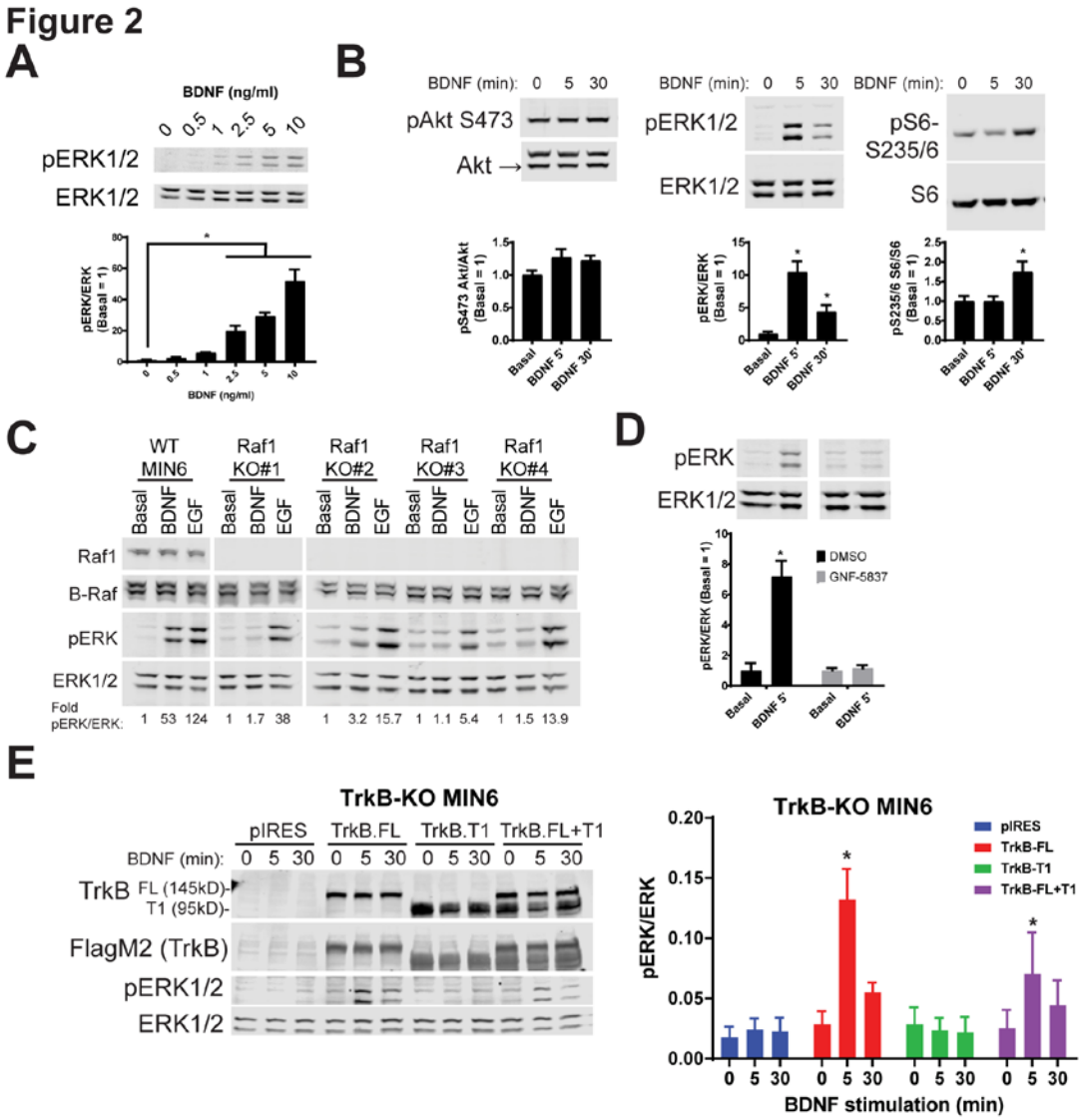

Figure 2 TrkB is necessary and sufficient for BDNF signaling in MIN6 β cells. A) MIN6 cells were preincubated in glucose-free KRBH and then treated with increasing doses of BDNF for 5 min. pERK1/2 and ERK1/2 were immunoblotted and quantitation is show below as bar graphs. Data are the mean ± SE of three independent passages of cells. *, P<0.05. **B)** MIN6 cells were preincubated in KRBH with 2 mM glucose for 2h and then treated with or without 10 ng/ml BDNF for 5 and 30 min. Data are the mean ± SE of three independent experiments. *, P<0.05. **C)** Wild type and Raf1 knockout MIN6 cells were preincubated in KRBH without glucose for 2 h and then stimulated with either 10 ng/ml BDNF or EGF for 5 min. Fold pERK1/2/ERK1/2 responses are shown for 4 different Raf1 KO clones. Vertical white lines indicate intervening lanes were removed. **D)** MIN6 cells were preincubated in KRBH with 2 mM glucose for 2 h and stimulated with 10 ng/ml BDNF for 5 min. DMSO or 10 μM GNF-5837 was added 30 min prior to the stimulation. **E))** TrkB KO MIN6 cells were transfected with plasmids expressing full-length TrkB (TrkB-FL), TrkB-T1, both, or empty vector. After 48 h cells were preincubated in KRBH with 4.5 mM glucose for 2 h and stimulated with 10 ng/ml BDNF for 5 and 30 min. Data are the mean ± SE of three independent experiments. *, P<0.05 for 0 vs 5 min of BDNF stimulation by two-way ANOVA using Dunnet's multiple comparisons test.

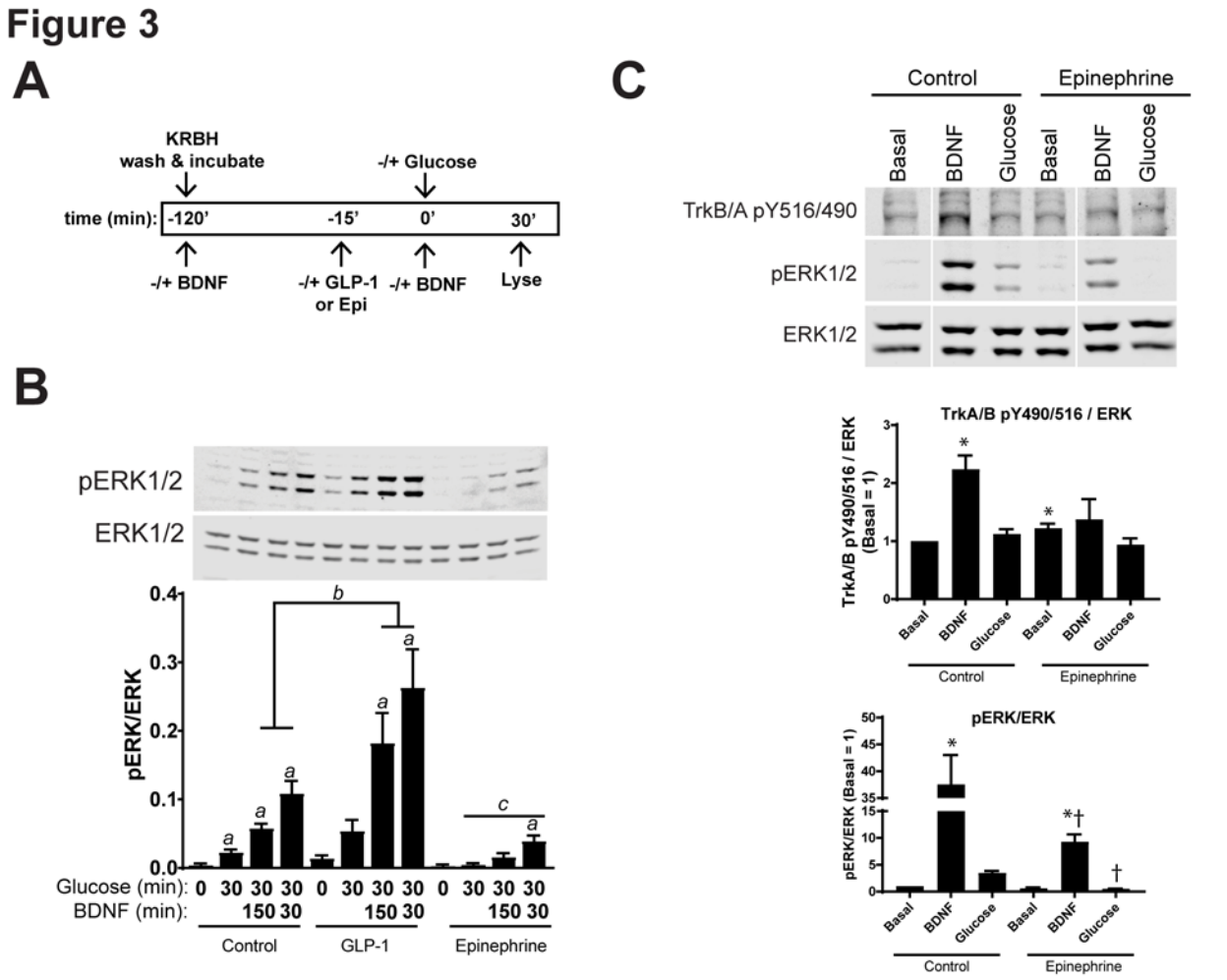

Figure 3. BDNF-TrkB exhibits crosstalk with GLP-1 and adrenergic signaling pathways in the β cell. A) Pictorial diagram of cell treatments for Fig. 3B. **B)** MIN6 cells were preincubated in glucose-free KRBH for 2 h with or without the addition of 10 ng/ml BDNF (150 min final treatment). Prior to the glucose addition cells were treated with or without 50 nM GLP-1 or 5 μM epinephrine for 15 min. After 2 h, 20 mM glucose was added either alone, to the BDNF pre-treated cells, or in combination with BDNF for 30 min. Phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 immunoblots are shown and the quantitated ratio of pERK/ERK is shown below. Data are the mean ± SE of three independent experiments. *a*, P<0.05 vs 0 min glucose, respectively. *b*, P<0.05 control vs GLP-1. *c*, P<0.05 control vs epinephrine. **C)** MIN6 cells were preincubated in KRBH without glucose for 2 h. Cells were pretreated for 15 min with 10 μM epinephrine and then stimulated with 10 ng/ml BDNF or 20 mM glucose for 5 min. Data are the mean ± SE of three independent experiments. Vertical white lines indicate intervening lanes were removed. *,P<0.05 vs basal. †,P<0.05 control vs epinephrine.

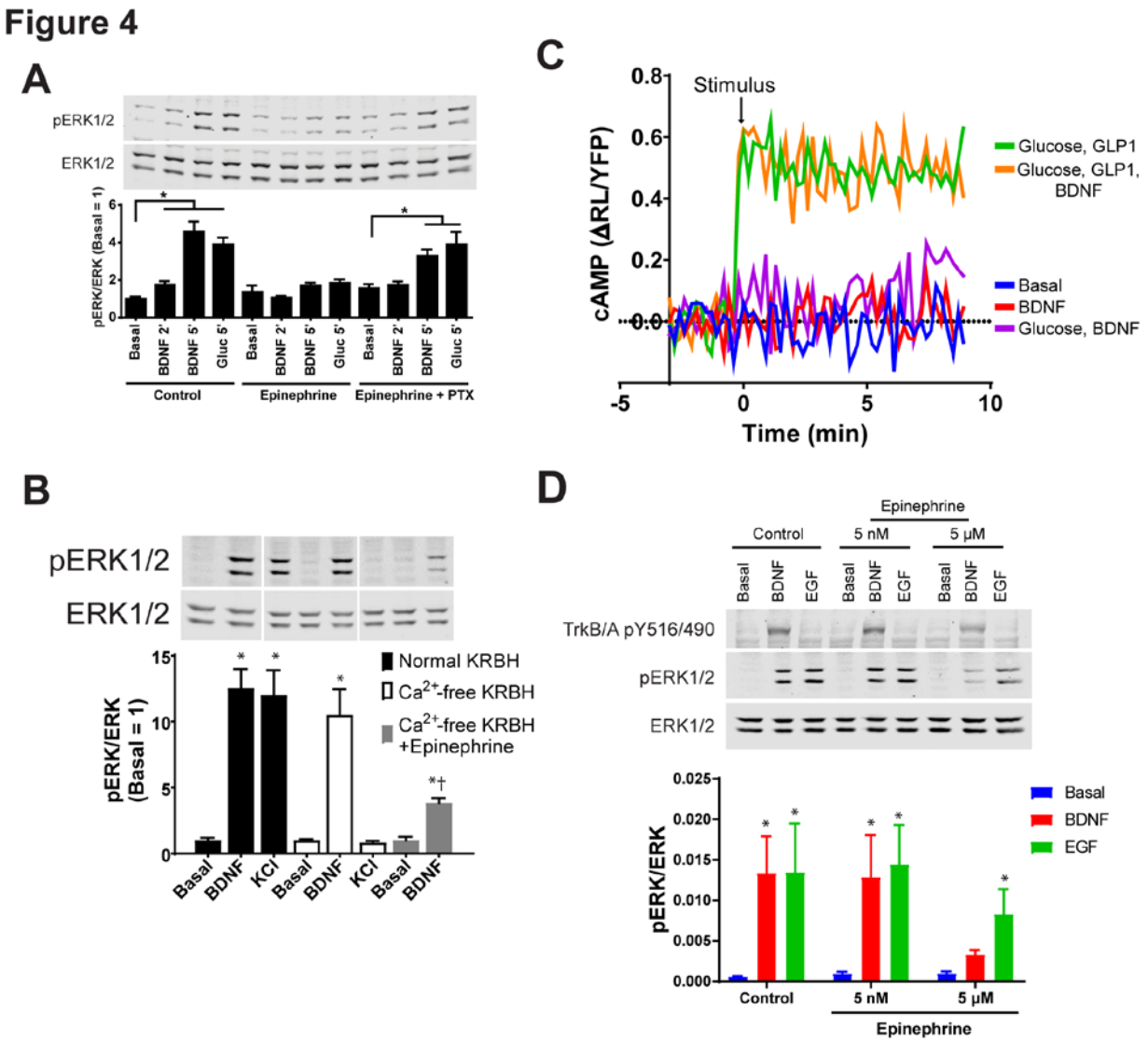

Figure 4. α_2-adrenergic receptor activation blocks BDNF stimulated ERK1/2 activation through Gαi independent of Ca^{2+} influx and cAMP generation. A) INS1 β cells were treated with 200 ng/ml pertussis toxin (PTX) for 18 h in culture medium. Cells were then incubated in KRBH with 2 mM glucose in the continued presence or absence of PTX for 2 h. Prior to stimulation, cells were treated with or without 10 μM epinephrine for 15 min. Cells were stimulated with or without 10 ng/ml BDNF or 20 mM glucose for the indicated time. Data are the mean ± SE of three independent experiments. *, P<0.05. **B)** MIN6 cells were preincubated in normal (Ca^{2+}-containing) or Ca^{2+}-free KRBH (compensated with additional 2 mM $MgCl_2$) without glucose for 2 h. 15 min prior to stimulation 10 μM epinephrine was added where indicated. Cells were stimulated with 10 ng/ml BDNF or 50 mM KCl for 5 min. Data are the mean ± SE. *, P<0.05 compared to respective basal. †,P<0.05 compared to normal KRBH. C**)** Intracellular cAMP generation was measured using MIN6 CAMYEL cells. Cells were preincubated in KRBH without glucose for 1.5 h and treated with 20 μM coelenterazine for 20 min. After a 3 min baseline measurement, stimuli were injected and BRET signal was monitored. Stimuli concentrations were: 20 mM Glucose, 30 nM GLP-1, 10 ng/ml BDNF. Data are representative of three independent experiments. **D)** MIN6 cells were preincubated in glucose-free KRBH for 2 h and then stimulated for 5 min with 10 ng/ml EGF or 10 ng/ml BDNF. Prior to stimulation, cells were treated with or without 5 μM

or 5 nM epinephrine for 15 min. Data represent the mean ± SD of three independent experiments. *, $P<0.05$ vs Basal Control by two-way ANOVA with Dunnett's multiple comparisons test.

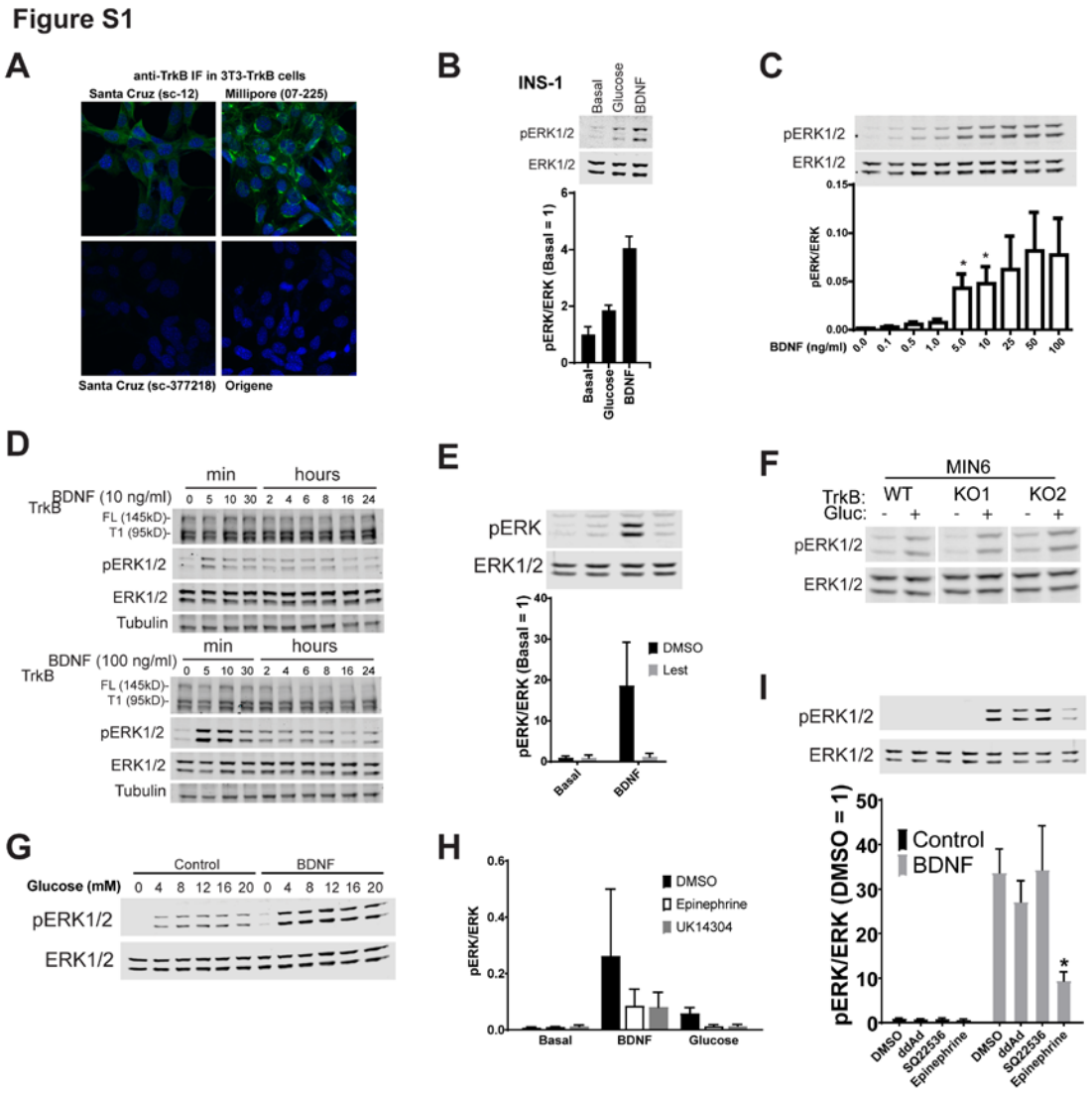

Figure S1. A) Anti-TrkB immunofluorescent staining in 3T3-TrkB cells to validate antibody suitability. **B)** INS1 cells were preincubated 2 h in glucose-free KRBH and then stimulated with glucose (20 mM) or BDNF (10 ng/ml) for 5 min. **C)** MIN6 cells were preincubated for 2 h in KRBH before stimulating with different doses of BDNF for 5 min. **D)** Time course of BDNF treatment in MIN6 cells at 10 and 100 ng/ml. High dose BDNF leads to TrkB-FL degradation. **E)** MIN6 cells treated as in (**C**) were treated with lestaurtinib (2 μM) for 30 min prior to stimulation with BDNF (50 ng/ml). **F)** Wild type and two distinct clonal lines of TrkB KO (KO1, KO2) MIN6 cells were preincubated in KRBH without glucose for 2 h and stimulated with 20 mM glucose for 5 min. Data are the mean ± SE of three experiments. **G)** MIN6 cells were preincubated in glucose-free KRBH for 2 h and then stimulated simultaneously with BDNF (10 ng/ml) and different doses of glucose for 30 min. Data are representative of three independent experiments. **H)** MIN6 cells were treated as in (**C**) with the addition of stimulation with 20 mM glucose for 5 min. Prior to BDNF (10 ng/ml) or glucose stimulation, cells were treated for 15 min with 0.1% DMSO, 10 μM epinephrine, or 10 μM UK14304. Data are the mean ± SD of two independent experiments. **I)** MIN6 cells were preincubated in KRBH without glucose for 2 h before stimulation with 10 ng/ml BDNF for 5 min. Prior to BDNF stimulation, cells were treated for 10 min with 0.1% DMSO, 100 μM dideoxyadenosine (ddAd), 100 μM SQ22536, or 5 μM epinephrine. Data are the mean ± SE of three independent experiments. *, P<0.05 compared to BDNF-stimulated DMSO control.

Antibody Details							
Antigen	**Host Species**	**kDa**	**Company**	**Cat#**	**Dilution for IB**	**Dilution for IF**	**Epitope**
pERK1/2	Mouse	42,44	Cell Signaling	9106	1:1000		
ERK1/2	Rabbit	42,44	In-house	Y691	1:3000		
TrkB	Rabbit	145, 95	Millipore	07-225	1:1000	1:100	entire extracellular domain of rat TrkB
TrkB	Rabbit	145, 95	Abcam	ab134155	-	1:100	Synthetic peptide (between 130 and 160aa in human trkB) (internal sequence)
TrkB	Rabbit	145, 95	Santa Cruz	sc-12	-	1:100	C-terminal cytoplasmic domain of mouse TrkB, peptide should match human not rat.
TrkB	Mouse	145, 95	Origene	TA500386	-	1:100	
TrkB	Mouse	145, 95	Santa Cruz	sc-377218		1:100	Anti-human
pY490/516 TrkA/B	Rabbit	145	Cell Signaling	4619	1:1000		
Insulin	Goat		Santa Cruz	sc-7839	-	1:300	
Glucagon	Mouse		Sigma	G2654	-	1:1000	
Akt	Mouse	60	Cell Signaling	2967	1:1000		
pS473 Akt	Rabbit	60	Cell Signaling	4060	1:1000		
Raf1	Rabbit	74	Santa Cruz	sc-133	1:200		
B-Raf	Mouse	86	Santa Cruz	sc-5284	1:250		
FlagM2	Mouse		Sigma	F1084	1:1000		
Secondary Antibodies for Licor Immunobloting							
donkey anti-rabbit 680 (red)					1:10,000		
donkey anti-mouse 800 (green)					1:10,000		
Secondary Antibodies for immunofluorescence							
d anti R 647					1:400		
d anti G 555					1:400		
d anti M 488					1:400		

Table S1. All antibodies for western blotting were diluted in Licor Blocking buffer (diluted 1:5 in TBS-T). Primary incubation was overnight. Secondary incubation was for 1h. Blots were washed 3 times for 10min each after primary and secondary. For immunofluorescence all blocking was 1h at RT, primary overnight at 4C and secondary 1h RT. All washing was 3 times for 10min each in PBS-T at RT.

Figure S2

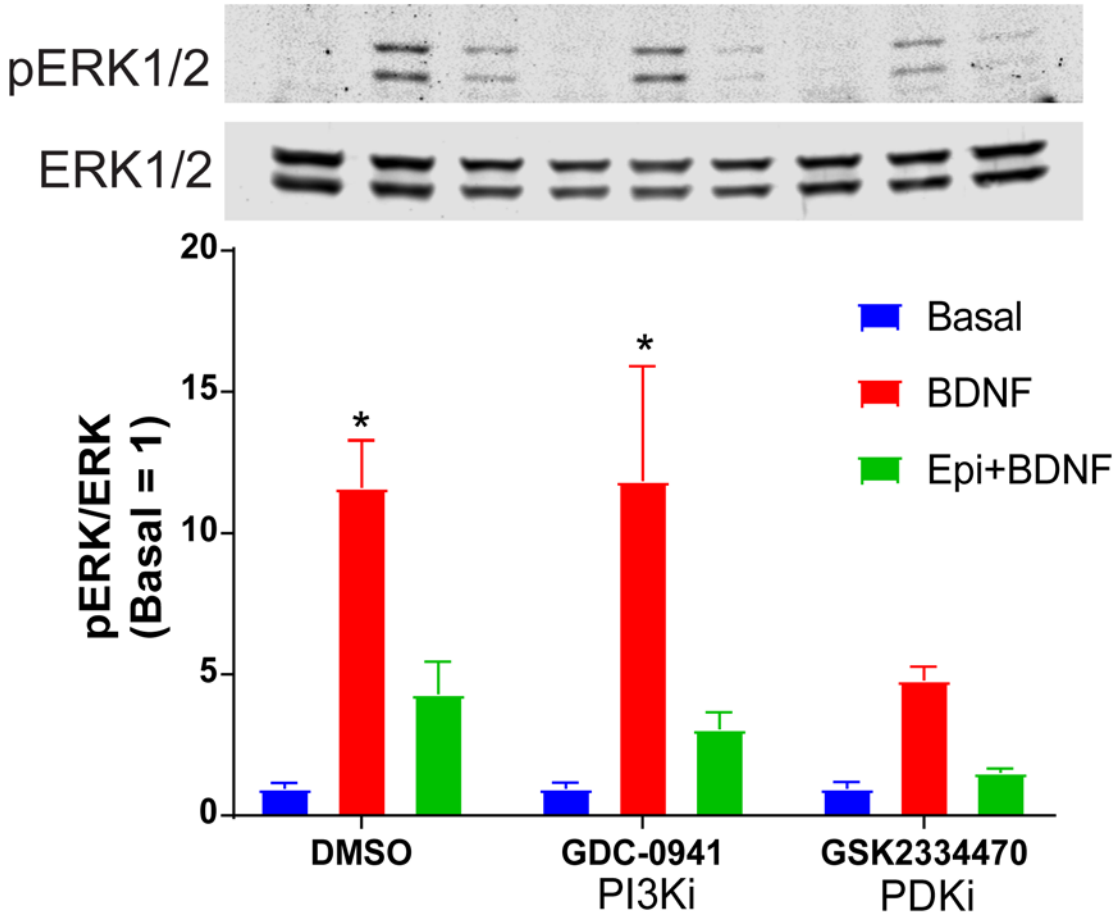

Figure S2. PI3K and PDK1 inhibitors do not prevent epinephrine-mediated inhibition of BDNF signaling in β cells. MIN6 cells were preincubated for 1.5 h in glucose-free KRBH and then treated with DMSO (0.1%), GDC-0941 (250 nM) or GSK2334470 (250 nM) for 15 min. Cells were then treated or not with 5 μM epinephrine for 15 min before stimulation with BDNF (10 ng/ml) for 5 min. Bar graph represents the mean ± SE for four independent experiments. *,P<0.05 vs. Basal by two-way ANOVA with Dunnett's multiple comparisons test.