NMDA receptors mediate leptin signaling and regulate potassium channel trafficking in pancreatic β-cells

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Yi Wu[‡1], **Dale A. Fortin**[‡1], **Veronica A. Cochrane**[‡2], **Pei-Chun Chen**[§3], **and Show-Ling Shyng**[‡4]

From the [‡]*Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon 97239 and the* [§]*Department of Physiology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan*

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NMDA receptors (NMDARs) are Ca^{2+}-permeant, ligand-gated ion channels activated by the excitatory neurotransmitter glutamate and have well-characterized roles in the nervous system. The expression and function of NMDARs in pancreatic β-cells, by contrast, are poorly understood. Here, we report a novel function of NMDARs in β-cells. Using a combination of biochemistry, electrophysiology, and imaging techniques, we now show that NMDARs have a key role in mediating the effect of leptin to modulate β-cell electrical activity by promoting AMP-activated protein kinase (AMPK)-dependent trafficking of K_{ATP} and Kv2.1 channels to the plasma membrane. Blocking NMDAR activity inhibited the ability of leptin to activate AMPK, induce K_{ATP} and Kv2.1 channel trafficking, and promote membrane hyperpolarization. Conversely, activation of NMDARs mimicked the effect of leptin, causing Ca^{2+} influx, AMPK activation, and increased trafficking of K_{ATP} and Kv2.1 channels to the plasma membrane, and triggered membrane hyperpolarization. Moreover, leptin potentiated NMDAR currents and triggered NMDAR-dependent Ca^{2+} influx. Importantly, NMDAR-mediated signaling was observed in rat insulinoma 832/13 cells and in human β-cells, indicating that this pathway is conserved across species. The ability of NMDARs to regulate potassium channel surface expression and thus, β-cell excitability provides mechanistic insight into the recently reported insulinotropic effects of NMDAR antagonists and therefore highlights the therapeutic potential of these drugs in managing type 2 diabetes.

Insulin secretion by β-cells is under the control of a complex network of ion channels and signaling events (1). ATP-sensitive potassium (K_{ATP})[5] channels composed of Kir6.2 and sulfonylurea receptor 1 (SUR1) subunits have a key role by coupling glucose metabolism to the β-cell membrane potential (2). Upon glucose stimulation, K_{ATP} channels close in response to an increased intracellular ATP to ADP ratio, resulting in membrane depolarization, which activates voltage-gated calcium channels; the ensuing calcium influx then triggers insulin release (2, 3). Cessation of insulin secretion occurs when the β-cell membrane potential returns to a hyperpolarized resting state. An important contributor of β-cell membrane repolarization is the voltage-gated delayed rectifier potassium channel Kv2.1. Reduction of Kv2.1 function in β-cells has been shown to enhance action potential duration, calcium influx, and insulin secretion (4, 5). Recent studies showed that the density of K_{ATP} and Kv2.1 channels in β-cells is dynamically regulated by metabolic or hormonal signals to modulate cell excitability (6–10). In particular, leptin, a satiety hormone secreted by adipocytes to maintain energy and glucose homeostasis, was reported to promote trafficking of K_{ATP} channels (6, 8) and Kv2.1 channels (10) to the β-cell surface. Evidence suggests that leptin activates the AMP-activated protein kinase (AMPK) via its upstream kinase Ca^{2+}-calmodulin-dependent protein kinase kinase β (CaMKKβ) (8, 10); however, the mechanism by which leptin activates the CaMKKβ–AMPK pathway in β-cells is unclear.

NMDA receptors (NMDARs) are ionotropic glutamate receptors whose activation requires the co-agonists glutamate and glycine as well as membrane depolarization, which removes external Mg^{2+} block (11). NMDARs are Ca^{2+}-permeable, which endows them the ability to trigger Ca^{2+}-dependent signaling events. For example, in hippocampal neurons, Ca^{2+} influx through NMDARs is coupled to activation of the Ca^{2+}-dependent protein kinase CaMKK to induce long-term potentiation (12). Expression of NMDARs in β-cells has been reported since the mid-nineties (13–15). However, in contrast to their well-characterized functional role in the nervous system (16), the role of NMDARs in β-cells has remained elusive or even controversial. A recent study reported that inhibition of NMDARs *in vitro* and *in vivo* elicits increases in glucose-stimulated insulin secretion (GSIS) (17), but the underlying mechanism has yet to be elucidated.

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[1] Both authors contributed equally to this work.

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[3] To whom correspondence may be addressed: Dept. of Physiology, College of Medicine, National Cheng Kung University, No. 1 University Rd., Tainan 701, Taiwan. Tel.: 886-6-235-3535 (ext. 5423); Fax: 886-6-236-2780; E-mail: pcchen@mail.ncku.edu.tw.

[4] To whom correspondence may be addressed: Dept. of Biochemistry and Molecular Biology, School of Medicine, Oregon Health and Science University, 3181 S. W. Sam Jackson Park Rd., Portland, OR 97239. Tel.: 503-494-2694; Fax: 503-494-3849; E-mail: shyngs@ohsu.edu.

[5] The abbreviations used are: K_{ATP}, ATP-sensitive potassium; NMDAR, NMDA receptor; SUR1, sulfonylurea receptor 1; AMPK, AMP-activated protein kinase; CaMKK, Ca^{2+}-calmodulin-dependent protein kinase kinase; GSIS, glucose-stimulated insulin secretion; D-APV, D-(−)-2-amino-5-phosphonopentanoic acid; DXM, dextromethorphan; pAMPK, phosphorylated AMPK.

In the present study, we demonstrate that NMDARs are expressed by β-cells and are required for leptin-induced calcium influx, AMPK activation, increased K_{ATP} and Kv2.1 channel surface expression, and reductions in β-cell membrane excitability. Moreover, we show that activation of NMDARs alone induces channel trafficking and reduces β-cell membrane excitability. These findings reveal an important role of NMDARs in regulating β-cell excitability and provide a novel mechanistic paradigm for insulin secretion regulation.

Results

NMDARs are expressed in pancreatic β-cells

We previously reported that leptin increases the surface density of K_{ATP} and Kv2.1 channels in rat insulinoma INS-832/13 cells and human β-cells. In INS-832/13 cells, this increase is dependent upon activation of the AMPK, which is in turn dependent on its upstream effector, CaMKKβ (6, 10). Studies in hippocampal neurons have linked calcium influx through NMDARs to activation of the CaMKKβ–AMPK pathway (18, 19). Furthermore, NMDAR stimulation has been shown to increase K_{ATP} currents in an AMPK-dependent manner in subthalamic neurons (20, 21). These reports prompted us to investigate whether NMDARs could be involved in the leptin signaling pathway that regulates surface expression of K_{ATP} and Kv2.1 channels in β-cells.

Although expression of NMDARs and their functional roles have been studied in a number of rodent β-cell lines or primary islets by measuring mRNA, protein, or currents, the results vary and in some cases are controversial (13, 15, 17, 22–24). We first determined whether NMDARs are expressed by INS-832/13 cells, which were used in our previous studies. Immunoblotting was used to probe the NMDAR subunit GluN1, which is the mandatory subunit for all functional NMDARs (25), in INS-832/13 cell lysate. Although GluN1 protein was expressed by INS-832/13 cells, its expression level was less than that observed in whole brain homogenate (Fig. 1*A*). No expression was observed in COS cells, which lack NMDARs. Immunostaining illustrated that only 43% of insulin-positive INS-832/13 and 46% of human β-cells expressed detectable levels of GluN1 protein (Fig. 1*B*). Notably, although most GluN1-positive INS-832/13 and human β-cells showed low level of staining, some INS-832/13 cells showed intense GluN1 signals (Fig. 1, *C* and *D*). In addition, we found that a small percentage of dissociated islet cells (23%), although positive for GluN1, were not identified as β-cells, implicating that other cell types within human islets also express NMDARs.

We next conducted whole-cell patch clamp recordings and used local pressure (puff) application of NMDA (1 mM) to assess NMDAR function. In 10 of 21 cells tested, puff application of NMDA induced inward currents (holding potential, −70 mV; no external Mg^{2+}) with a mean of 9.0 ± 1.4 pA that was inhibited to 1.8 ± 0.2 pA by the non-competitive NMDAR antagonist MK-801 (50 μM; $p < 0.001$, $n = 10$ by paired *t* test; Fig. 1*E*). Application of the competitive NMDAR antagonist D-APV (50 μM) also reduced NMDAR currents (from 28.4 ± 7.2 to 9.7 ± 5.2 pA; $p < 0.001$, $n = 12$ by paired *t* test; not shown). Consistent with immunostaining results, not all cells recorded had detectable NMDAR currents, and those that did displayed a range of amplitudes that reflected the heterogeneity in NMDAR expression (Fig. 1*D*). Importantly, NMDA-evoked currents were also observed in dispersed human β-cells and were reduced by MK-801 (from 21.3 ± 8.9 to 6.3 ± 3.0 pA; $p < 0.001$, $n = 5$ by paired *t* test; Fig. 1*F*). Puff application of glutamate (1 mM), a physiological ligand of the NMDAR, also elicited outward currents when cells were held at a positive potential of 40 mV that were reversibly blocked by MK-801 (27.8 ± 7.0 pA for glutamate, 2.3 ± 1.4 pA for MK-801, and 23.5 ± 6.7 pA for MK-801 washout; $p < 0.01$, $n = 5$ by paired *t* test; Fig. 1*G*). Together, these results show that both INS-832/13 cells and human β-cells express functional NMDARs.

NMDARs are required for leptin-induced surface trafficking of K_{ATP} and Kv2.1 channels

To test the role of NMDARs in leptin-induced surface trafficking of K_{ATP} and Kv2.1 channels, we monitored surface expression of these channels using surface biotinylation following treatment of INS-832/13 cells with 0.1% DMSO, 10 nM leptin, or 50 μM NMDA for 30 min in the absence or presence of MK-801. To test for specificity among ionotropic glutamate receptors, the effect of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (50 μM), an agonist of the ionotropic AMPA receptor, which has been reported to be expressed in β-cells (26, 27), was also examined in the absence or presence of MK-801. As expected (6), leptin induced a significant increase (~2-fold) in surface SUR1 as compared with controls (Fig. 2*A*). Strikingly, although treatment with NMDA induced a similar increase in biotinylated SUR1 protein, treatment with AMPA did not, suggesting that this is not a general response to ionotropic glutamate receptor activation (Fig. 2*A*). Both leptin- and NMDA-induced increases in biotinylated SUR1 and Kv2.1 were inhibited by NMDAR antagonists (Fig. 2, *A* and *C*). Although a previous study attributed the leptin-induced trafficking of K_{ATP} channels to activation of TRPC4 channels (8), we failed to see an effect of TRPC4 inhibition (ML204; 10 μM) (28) on leptin-induced increases in surface expression of either K_{ATP} or Kv2.1 channels (Fig. 2, *B* and *D*).

Previously, we and others showed that leptin increases AMPK phosphorylation at residue Thr-172 of the catalytic subunit, a signature of AMPK activation (8, 10). The increase in phosphorylation was shown to be Ca^{2+}-dependent and required the Ca^{2+}-dependent kinase CaMKKβ. Importantly, activation of AMPK was required for the trafficking of K_{ATP} and Kv2.1 channels (7). We found that, like leptin, NMDA also increased AMPK Thr(P)-172 and that the increase was blocked by MK-801 in both cases, implying that they activate similar signaling pathways (Fig. 3, *A* and *B*). Taken together, these results reveal a necessary role of NMDARs in mediating the effect of leptin on AMPK activation and the subsequent surface expression of K_{ATP} and Kv2.1 channels.

NMDARs mediate leptin-induced hyperpolarization in INS-832/13 cells

Delivery of K_{ATP} channels to the plasma membrane of INS-832/13 cells upon leptin signaling results in membrane hyperpolarization (10). We reasoned that this hyperpolarization should

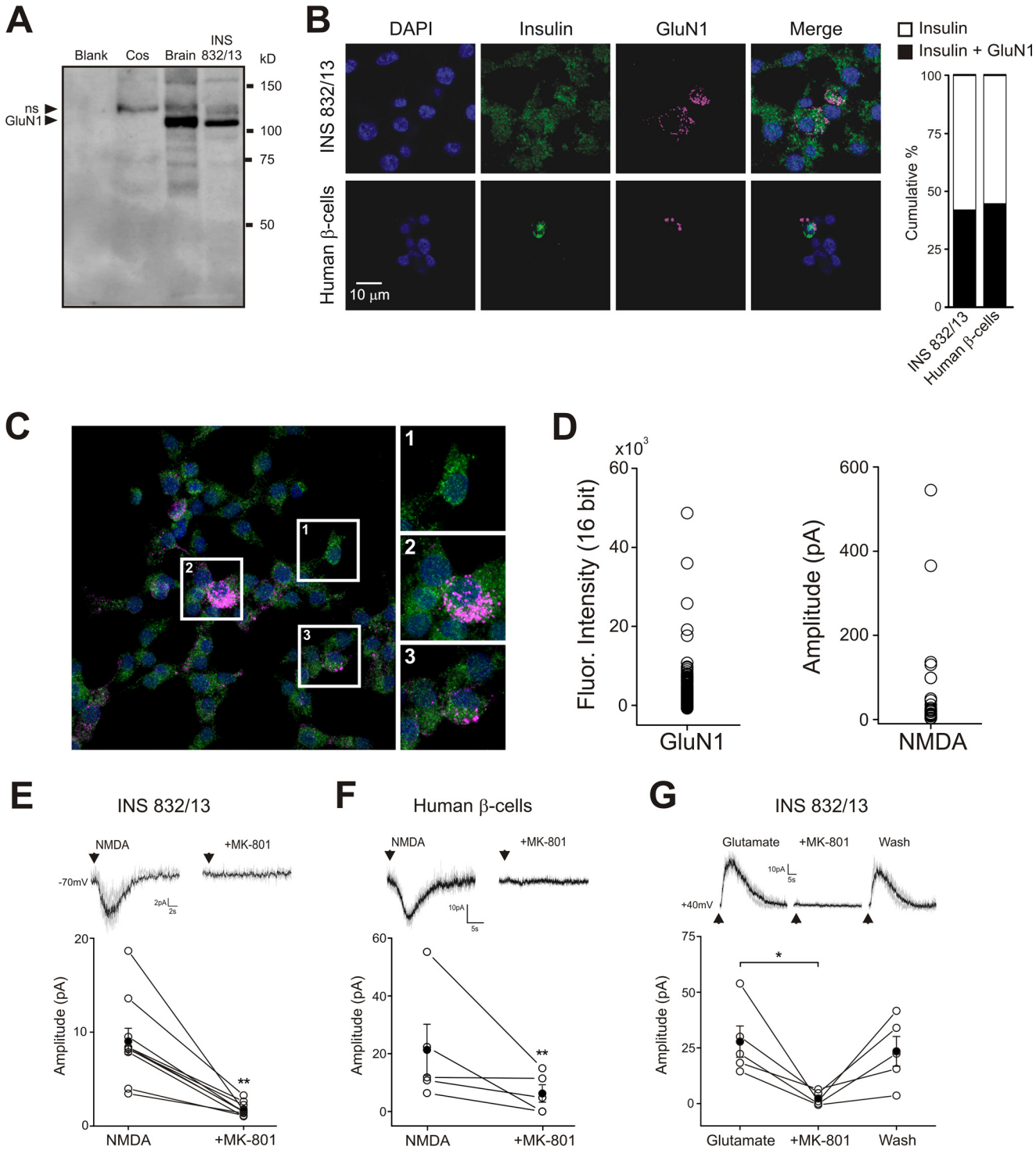

Figure 1. INS-832/13 and human β-cells express functional NMDARs. *A*, immunoblot showing expression of GluN1 protein (105 kDa) in whole brain and INS-832/13 cell lysate but not in lysate prepared from COS cells (*ns*, nonspecific protein band). *B*, confocal image of INS-832/13 cells (*top panels*) and human β-cells (*lower panels*) immunostained for DNA (*DAPI*; *blue*), insulin (*green*), and GluN1 (*magenta*). *Right*, graph showing the percentage of INS-832/13 and human β-cells that expressed GluN1 protein (INS-832/13, 144 of 339 cells; human β-cells, 136 of 296 cells from two donors). *C*, immunofluorescence image of INS-832/13 cells immunostained for insulin (*green*), GluN1 (*magenta*), and DNA (DAPI; *blue*). Zoomed-in view of *numbered boxed regions* is shown to the *right* for cells with no (*1*), high (*2*), and low (*3*) expression of GluN1. *D*, distributions of single-cell integrated fluorescence (*Fluor.*) intensities of GluN1 (*left*; $n = 282$) and single-cell NMDA-induced current amplitudes (*right*; $n = 32$). *E* and *F*, whole-cell voltage clamp traces (holding potential at −70 mV) recorded from a single INS-832/13 cell (*E*) or human β-cell (*F*) in response to NMDA (1 mM puff) and after the subsequent addition of NMDAR antagonist MK-801 (50 μM). *G*, whole-cell voltage clamp traces (holding potential at +40 mV) recorded from a single INS-832/13 cell in response to glutamate (1 mM puff) and subsequent addition of MK-801 and 10 min after MK-801 washout (*Wash*). For experiments shown in *E–G*, the external Tyrode's solution was supplemented with 0.1 mM glycine but no Mg^{2+} or glucose. In *E–G*, *black* traces represent the mean response to three consecutive puff-evoked currents shown in *gray*. *Arrows* denote time of puff. Group data for mean (*filled circles*) and individual (*open circles*) changes in the amplitude of puff-evoked currents are plotted below. *Error bars* represent S.E. (see "Experimental procedures"). *, $p < 0.01$; **, $p < 0.001$.

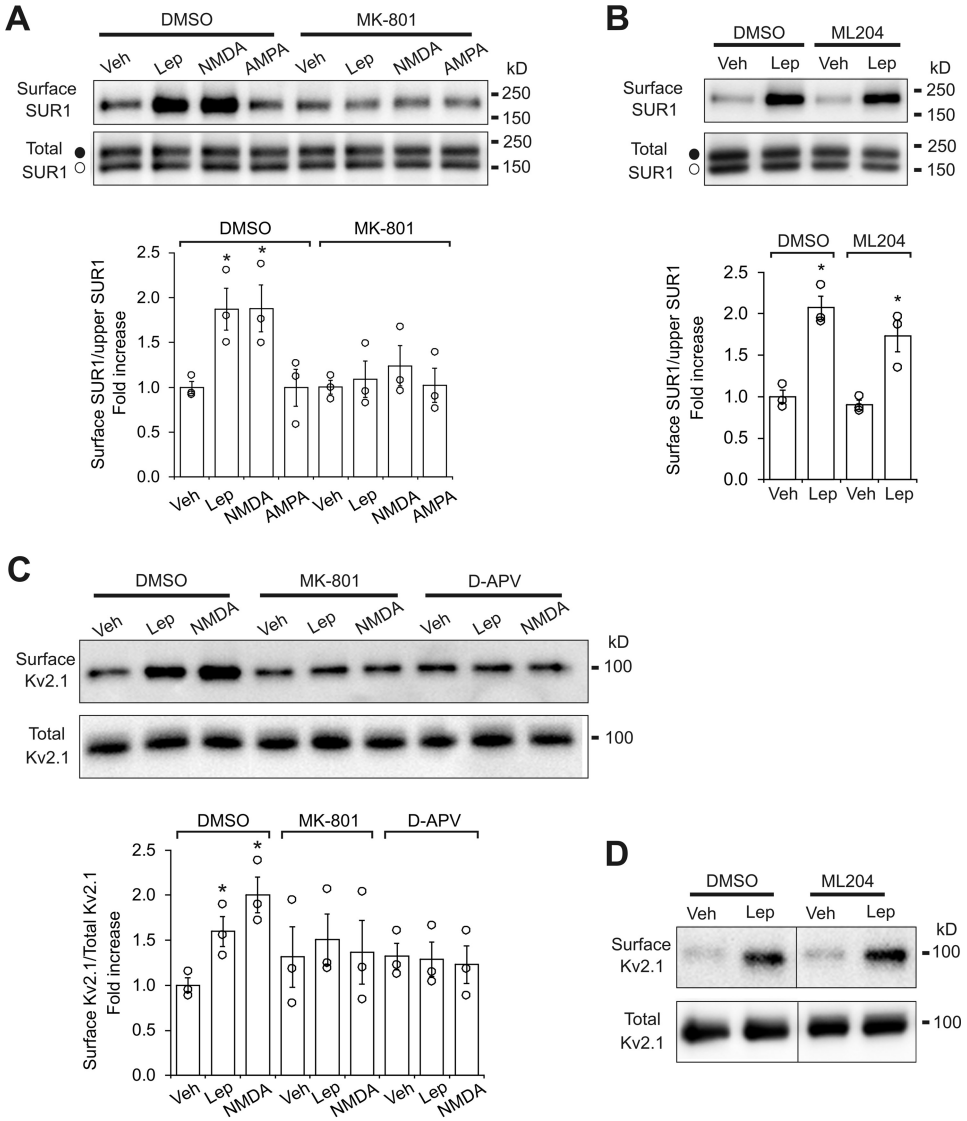

Figure 2. Ionotropic function of NMDARs is required for leptin-induced trafficking of SUR1 (K_{ATP} channels) and Kv2.1. *A*, representative immunoblot showing surface-biotinylated SUR1 protein (*upper* blot) and total SUR1 protein (*lower* blot) from INS-832/13 cells pretreated with 0.1% DMSO, 10 nM leptin (*Lep*), 50 μM NMDA, and 50 μM (*R*,*S*)-AMPA in the absence or presence of 50 μM MK-801. Total SUR1 antibody recognizes both complex-glycosylated mature (*filled circles*) and core-glycosylated immature (*open circles*) SUR1 protein. Group data are shown below from three independent experiments. *, $p < 0.05$. *B*, representative immunoblot showing that the increase in surface SUR1 protein was not prevented by the TRPC4 inhibitor ML204 (10 μM). Group data are shown below from three independent experiments. *Error bars* in bar graphs shown in *A*, *B*, and *C* represent S.E. *, $p < 0.05$. *C*, representative immunoblot showing surface-biotinylated Kv2.1 protein (*upper* blot) and total Kv2.1 protein (*lower* blot) from INS-832/13 cells pretreated with DMSO, leptin, or NMDA in the absence or presence of MK-801 (as in *A*) or the competitive NMDAR antagonist D-APV (50 μM). Group data from three independent experiments are shown below. *, $p < 0.05$. *D*, example immunoblot showing that inhibition of TRPC4 with ML204 did not alter the increase in Kv2.1 surface trafficking by leptin. *Veh*, vehicle. The *thin vertical line* in each blot separates two parts of the same blot.

also be NMDAR-dependent. To monitor changes in membrane potential, we used cell-attached patch clamp recording, which provides an accurate measure of membrane potential, maintains cell integrity, and prevents dialysis of soluble factors that may be important for proper signaling (29). Membrane potential was recorded in response to vehicle or leptin in the absence or presence of tolbutamide, an inhibitor of K_{ATP} channels, the competitive NMDAR antagonist D-APV, or the TRPC4 inhibitor ML204. In addition, dextromethorphan (DXM), an NMDAR antagonist recently shown to stimulate insulin secretion and improve glucose tolerance in type 2 diabetes patients (17), was also tested.

In the majority of cells tested (23 of 32 cells), bath application of leptin in the presence of 11 mM glucose induced a significant membrane hyperpolarization ($\Delta V_m = -0.2 \pm 0.7$ and -23.1 ± 3.5 mV for vehicle and leptin, respectively; $p < 0.001$) (Fig. 4). Leptin induced similar results when cells were recorded under whole-cell mode ($\Delta V_m = -26.6 \pm 3.6$ mV; $n = 16$), confirming the integrity of our cell-attached recordings. The onset of hyperpolarization occurred ~1–10 min after the start of leptin perfusion and was largely abolished by co-application with tolbutamide (30) ($\Delta V_m = -4.7 \pm 2.0$ mV), indicating that the source of hyperpolarization was from K_{ATP} channels. These results are in good agreement with those reported previously in a different β-cell line, CR1-G1 (31). By contrast, in the absence of leptin, tolbutamide alone did not significantly alter the depolarized baseline potential of INS-832/13 cells bathed in 11 mM glucose (baseline, 3.6 ± 2.5 mV; tolbutamide, 7.9 ± 3.3 mV; $n =$

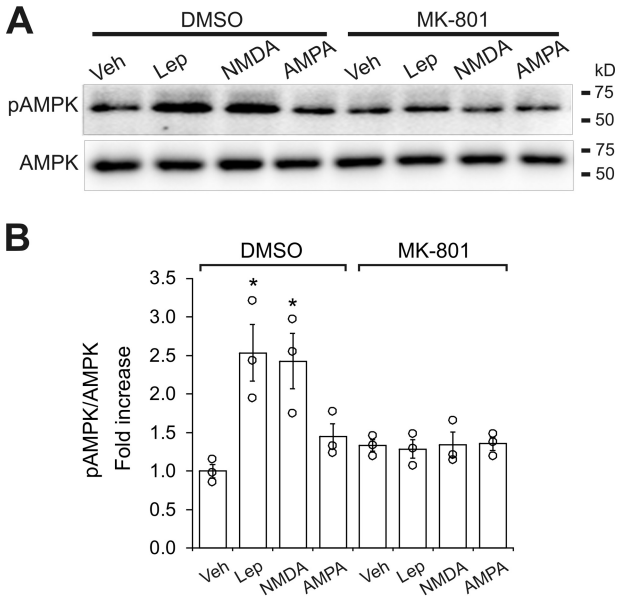

Figure 3. Leptin-induced phosphorylation of AMPK requires NMDARs. *A*, representative immunoblot probed for total and pAMPK (phosphorylated at Thr-172) protein from INS-832/13 cells treated with vehicle (*Veh*), leptin (*Lep*), NMDA, or AMPA in the absence or presence of MK-801 (concentrations as in Fig. 2*A*). *B*, -fold increase in the mean pAMPK/AMPK ratio for each condition from three independent experiments. *Error bars* represent S.E. *, $p < 0.05$.

9), indicating that under this condition most existing K_{ATP} channels are closed as expected. We next examined whether blockade of NMDARs could inhibit the ability of leptin to hyperpolarize the cell membrane. Application of either D-APV or DXM significantly reduced the level of hyperpolarization by leptin ($\Delta V_m = -6.2 \pm 2.9$ and -5.9 ± 2.1 mV, respectively; Fig. 4*C*). By contrast, cells receiving bath application of leptin with the TRPC4 inhibitor ML204 showed a similar level of hyperpolarization as with leptin alone ($\Delta V_m = -25.8 \pm 3.6$ mV; Fig. 4, *B* and *C*), suggesting that TRPC4 channels do not contribute significantly to leptin-induced membrane hyperpolarization.

NMDAR activation is sufficient to induce membrane hyperpolarization in β-cells

Because NMDA is sufficient to enhance surface expression of K_{ATP} channels, we asked whether it also regulates β-cell membrane excitability. In the majority of cells tested (~80%), perfusion of NMDA (50 μM) alone triggered a significant hyperpolarization in membrane potential in both INS-832/13 ($\Delta V_m = -20.7 \pm 4.4$ mV; $p < 0.01$) and human β-cells ($\Delta V_m = -30 \pm 5.4$ mV; $p < 0.001$) (Fig. 5*C*). The onset of hyperpolarization to NMDA treatment averaged ~7 min but ranged from 1 to 14 min in INS-832/13 cells and human β-cells after the start of perfusion. In some instances, NMDA induced a small membrane depolarization, indicative of NMDAR activation, prior to the onset of hyperpolarization (Fig. 5*A*, *lower* trace). Importantly, the NMDA-mediated hyperpolarization in both INS-832/13 and human β-cells was blocked by tolbutamide, suggesting that, like leptin, NMDA affects β-cell membrane potential via K_{ATP} channels (Fig. 5*C*). These data indicate that activation of NMDARs is sufficient to induce trafficking of K_{ATP} channels and subsequent membrane hyperpolarization in INS-832/13 and human β-cells.

We have shown previously that leptin increases AMPK phosphorylation via CaMKKβ, a known upstream kinase of AMPK, to regulate surface trafficking of Kv2.1 and K_{ATP} channels (10). Because NMDA also increased AMPK phosphorylation (Fig. 3), we tested whether the observed NMDAR-mediated hyperpolarization requires CaMKKβ. We found that although NMDA alone triggered a significant hyperpolarization in membrane potential ($\Delta V_m = -27.3 \pm 7.1$ mV; $n = 8$, $p < 0.05$), INS-832/13 cells pretreated with STO-609, a well known inhibitor of CaMKKβ, failed to hyperpolarize in the presence of NMDA (baseline, 6.8 ± 5.3 mV; NMDA, 3.1 ± 7.5 mV; $n = 6$) (Fig. 5, *D* and *E*). This result indicates that both leptin and NMDARs promote channel trafficking and INS-832/13 membrane hyperpolarization by CaMKKβ-dependent phosphorylation of AMPK.

Leptin causes increased Ca^{2+} influx through NMDARs

Given that CaMKKβ requires Ca^{2+} for its catalytic activity, we hypothesized that Ca^{2+} influx through NMDARs might be critical for leptin signaling. To directly monitor changes in intracellular [Ca^{2+}], β-cells were loaded with the cell-permeant fluorescent indicator dye Fluo-4 AM. Local puff application of NMDA caused a robust transient increase in intracellular [Ca^{2+}] in 70% of cells tested (Fig. 6, *A*, *B*, and *C*). On average, NMDA increased intracellular [Ca^{2+}] by 2.00 ± 0.26-fold. Importantly, the increase in intracellular [Ca^{2+}] by NMDA was abolished by co-application of either D-APV (1.02 ± 0.06-fold), MK-801 (1.12 ± 0.07-fold), or DXM (0.96 ± 0.03-fold) but not by ML204 (2.61 ± 0.64-fold) (Fig. 6*C*). These results demonstrate that NMDARs permit Ca^{2+} influx in INS-832/13 cells.

Next, we tested whether leptin induces Ca^{2+} influx through NMDARs. Bath application of leptin triggered a more sustained increase in intracellular Ca^{2+} as compared with puff application of NMDA (Fig. 6*D*); however, similar sustained increases in Ca^{2+} were observed when NMDA was bath-applied (see Fig. 6*D*, *inset*). On average, leptin induced a rise in intracellular Ca^{2+} that was 2.48 ± 0.15-fold higher than baseline. Importantly, this increase was markedly reduced by co-application of either D-APV (1.43 ± 0.07-fold), MK-801 (1.50 ± 0.08-fold), or DXM (1.28 ± 0.10-fold) but little affected by ML204 (2.32 ± 0.29-fold) (Fig. 6*E*). These data provide strong evidence that leptin triggers Ca^{2+} influx in INS-832/13 cells predominantly through NMDARs.

Leptin potentiates NMDAR currents in β-cells

In the hippocampus, leptin has been reported to facilitate long-term potentiation by enhancing NMDAR function (32, 33), raising the possibility that leptin may potentiate the function of NMDARs to trigger the downstream effects in β-cells. To test this possibility, we conducted whole-cell voltage clamp experiments from individual β-cells and induced NMDAR currents by puff application of NMDA before and after the subsequent addition of leptin. We found that NMDAR currents evoked in INS-832/13 cells were significantly potentiated by leptin (Fig. 7*A*, *left* trace). On average, leptin increased NMDAR currents by 164.2 ± 21.7% after 6 min (Fig. 7, *A* and *B*). The increase could be seen as quickly as 2 min following bath application of leptin and could be sustained for up to 30 min. In three

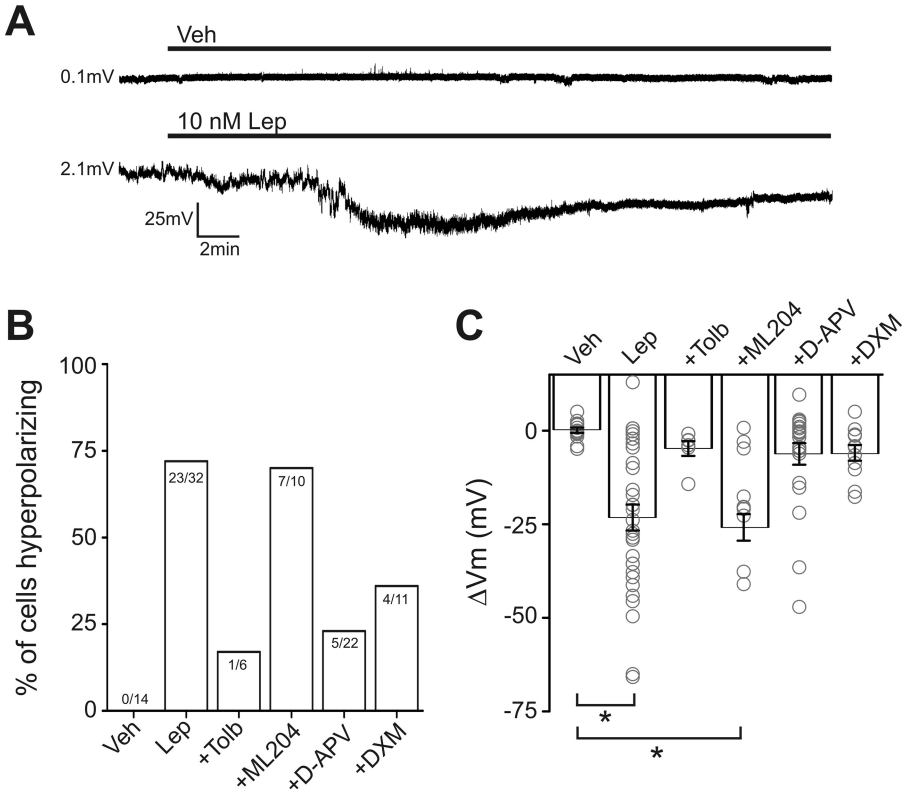

Figure 4. Leptin-induced hyperpolarization of INS-832/13 cells is dependent on K_{ATP} channels and NMDARs but not TRPC4/5 channels. *A*, cell-attached voltage traces recorded from INS-832/13 cells incubated in Tyrode's solution supplemented with 0.1 mM glycine and 11 mM glucose before and after perfusion of vehicle (*top* trace) or 10 nM leptin (*bottom* trace). The initial membrane potential for each cell, averaged over the first 2 min, is indicated to the *left* of each trace. *B*, bar graph depicting the percentage of cells that hyperpolarized in response to vehicle (*Veh*) only or leptin (*Lep*) in the absence or presence of the K_{ATP} channel inhibitor tolbutamide (*Tolb*; 300 μM), ML204 (10 μM), D-APV (50 μM), or DXM (10 μM). The number of cells tested is provided for each condition. The mean baseline potential across conditions was 1.5 ± 9.7 mV. *C*, group data illustrating the average degree of hyperpolarization for each condition. Data are shown as means (*bars*) along with individual cell responses (*open circles*). *Error bars* represent S.E. *, $p < 0.001$.

of eight cells, leptin failed to potentiate NMDAR currents, indicating that potentiation did not result from repeated NMDA puff applications (see example in Fig. 7*A*, *right* trace) and suggesting surface leptin receptor heterogeneity among β-cells. Importantly, leptin induced a marked enhancement in NMDA-evoked currents by 187.6 ± 32.7% in human β-cells (Fig. 7*C*). Taken together, these data reveal a novel link between leptin signaling and the function of NMDARs in β-cells.

Discussion

Utilizing a combination of biochemical, electrophysiological, and imaging approaches, we show that NMDARs play a crucial role in mediating the effect of leptin in β-cells. Together with our previous studies (6, 10), we propose a model by which leptin suppresses glucose-stimulated insulin secretion (Fig. 8). At 11 mM glucose under which condition β-cells are depolarized, NMDARs exposed to glutamate and glycine present in the extracellular milieu are able to conduct currents due to Mg^{2+} unblock. Leptin potentiates NMDAR currents, thus increasing Ca^{2+} influx to activate CaMKKβ, which then phosphorylates and activates AMPK to trigger PKA-dependent actin remodeling and trafficking of K_{ATP} and Kv2.1 channels to the cell surface. The increased surface expression of Kv2.1 channels induced by NMDAR activation is expected to shorten action potentials (10), facilitating membrane repolarization, whereas increased K_{ATP} channel abundance would facilitate membrane hyperpolarization to limit voltage-dependent Ca^{2+} influx and inhibit insulin secretion. The expression of functional NMDARs was observed in both rodent and human β-cells, indicating that this mechanism is highly conserved and likely plays an important role in regulating β-cell function and insulin release.

A recent study by Marquard *et al.* (17) found that NMDARs were required to limit GSIS and that inhibition of NMDARs could serve as a potential antidiabetic treatment. However, how NMDARs regulate insulin secretion remains unknown. Interestingly, these authors showed that inhibition of NMDARs by DXM could enhance GSIS without affecting basal insulin secretion; moreover, the effect of NMDAR inhibition on insulin secretion was absent in Kir6.2 knock-out mice, implicating involvement of K_{ATP} channels (17). Our findings provide a molecular mechanism for the observations made by Marquard *et al.* (17). Specifically, during low glucose, the negative resting β-cell membrane potential prevents NMDARs from opening due to blockade by external Mg^{2+} (11), rendering NMDAR inhibition ineffective. When glucose is high, β-cells depolarize, removing the Mg^{2+} block, allowing Ca^{2+} to enter and trigger channel trafficking, increasing K^+ efflux, and suppressing insulin release. Under this condition, inhibiting NMDARs would enhance insulin release by preventing K^+ channel trafficking.

The co-trafficking of K_{ATP} and Kv2.1 channels by leptin has been shown to require activation of CaMKKβ and its down-

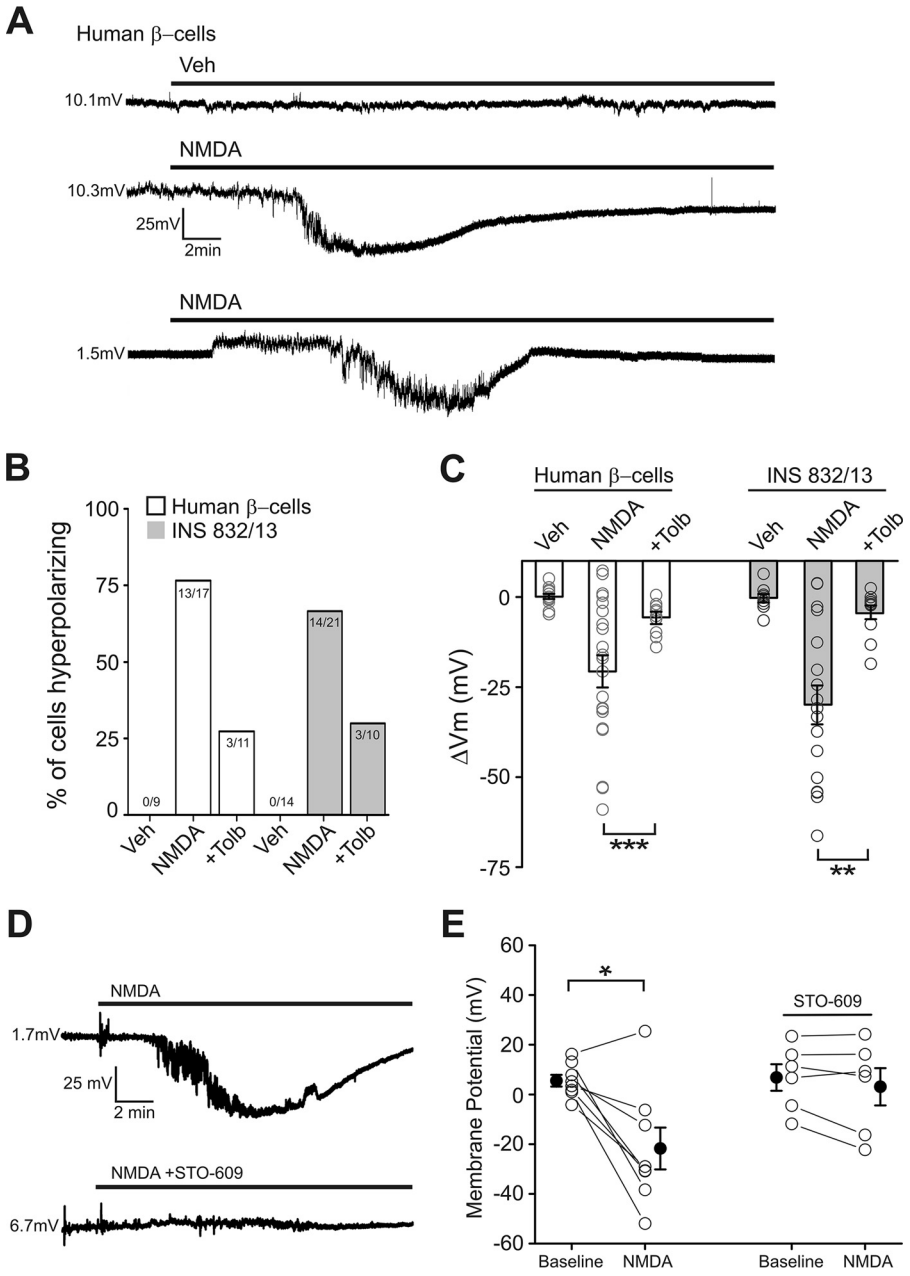

Figure 5. Activation of NMDARs is sufficient to induce K_{ATP}-dependent hyperpolarization in INS-832/13 and human β-cells. *A*, representative cell-attached voltage traces recorded from human β-cells incubated in Tyrode's solution supplemented with 0.1 mM glycine and 11 mM glucose before and after perfusion of vehicle (*top* trace) or 50 μM NMDA (*middle* and *bottom* traces). In a subset of cells tested, NMDA also induced a small membrane depolarization prior to the induction of hyperpolarization (*bottom* trace). The initial membrane potential for each cell, averaged over the first 2 min, is given to the *left* of each trace. *B*, bar graph depicting the percentage of human β-cells (*white filled bars*) and INS-832/13 cells (*gray filled bars*) that hyperpolarized in response to vehicle only or NMDA in the absence or presence of tolbutamide (*Tolb*). The number of cells tested is provided for each condition. *C*, group data illustrating the average degree of hyperpolarization observed in human β-cells and INS-832/13 cells for each condition. Data are shown as means (*bars*) along with individual cell responses (*open circles*). *Error bars* represent S.E. **, $p < 0.01$; ***, $p < 0.001$. *D*, example traces of NMDA-induced membrane hyperpolarization in an individual INS-832/13 cell in the absence (*top* trace) or presence of the CaMKKβ inhibitor STO-609 (10 μM; *bottom* trace). *E*, plot of individual changes (*open circles*) in membrane potential measured in INS-832/13 cells before and after bath application of NMDA in the absence ($n = 8$) or presence of STO-609 ($n = 6$). Means are indicated by *filled circles*. *Error bars* represent S.E. *, $p < 0.05$. *Veh*, vehicle.

stream effector AMPK (8, 10), which brings into question the potential source of Ca^{2+} influx upstream of CaMKKβ. Although it was reported previously that Ca^{2+} influx through TRPC4 channels was the most likely candidate (8), we found that TRPC4 inhibition was unable to prevent recruitment of K_{ATP} and Kv2.1 channels to plasma membrane. Rather, our data show that NMDARs are the primary Ca^{2+} source mediating the effect of leptin as NMDAR antagonists (competitive and non-competitive) as well as a selective pore blocker inhibited leptin-induced Ca^{2+} influx, activation of AMPK, K_{ATP} and Kv2.1 channel trafficking, and membrane hyperpolarization.

Our finding that NMDAR activation triggers the co-regulation of K_{ATP} and Kv2.1 channels highlights its importance in modulating the excitability of β-cells to control insulin secretion. This raises questions regarding how Kv2.1 and K_{ATP} channels are sorted within β-cells. For example, are they

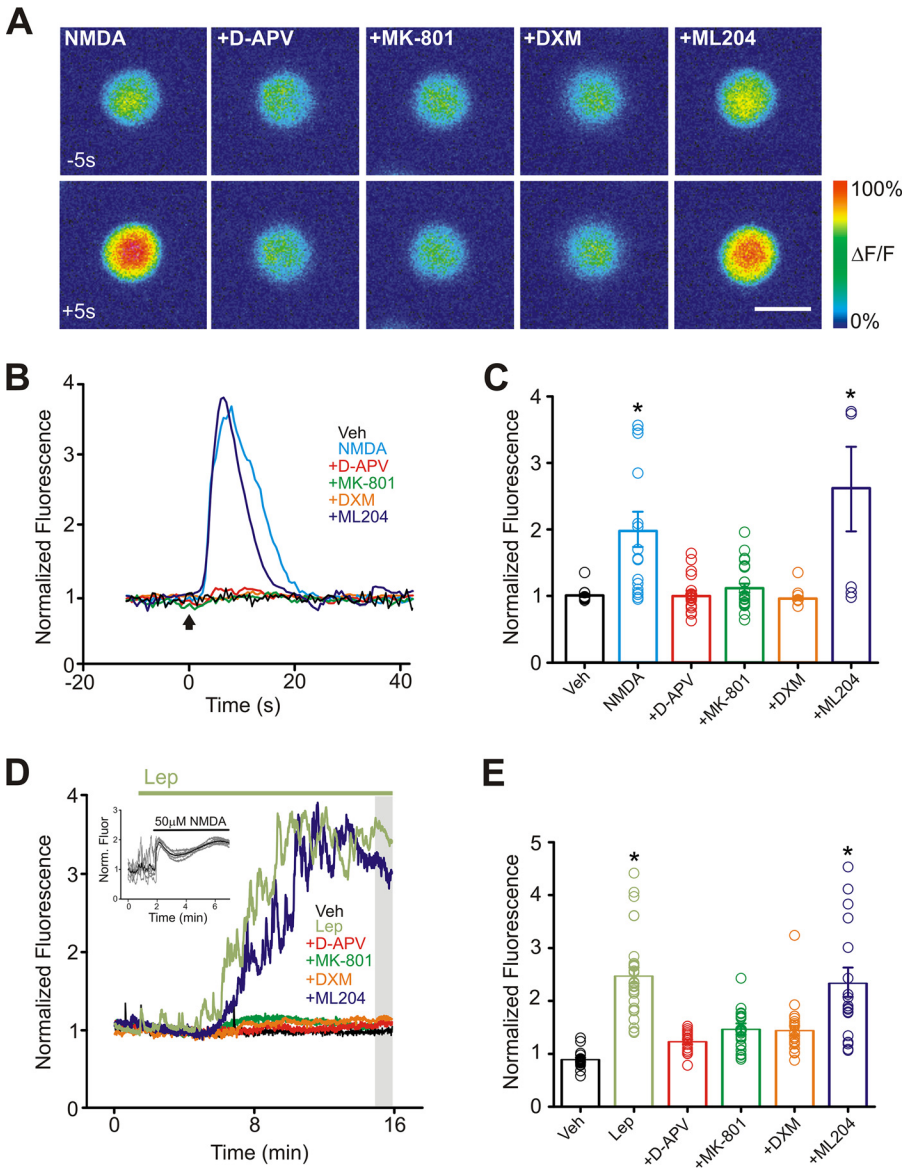

Figure 6. Leptin-induced Ca^{2+} increases recorded from INS-832/13 cells require NMDARs. *A*, Δ*F*/*F* pseudocolored images of individual INS-832/13 cells loaded with the Ca^{2+} indicator Fluo-4 AM. Cells were bathed in Tyrode's solution supplemented with 0.1 mM glycine but no Mg^{2+} or glucose during recording (see "Experimental procedures"). Images were taken 5 s before and 5 s after a puff of 100 μM NMDA in the absence or presence of D-APV (50 μM), MK-801 (50 μM), DXM (10 μM), or ML204 (50 μM). *Scale bar*, 10 μm. *B*, representative Ca^{2+} transients corresponding to the conditions shown in *A*. The *arrow* denotes time of the NMDA puff. *C*, group data showing the baseline-normalized changes in fluorescence for each condition. *Colored bars* correspond to representative Ca^{2+} transients shown in *B*. Data are shown as means (*bars*) along with individual cell responses (*open circles*). *Error bars* represent S.E. *, $p < 0.05$. *D*, representative Ca^{2+} transients recorded before and after bath application of 10 nM leptin (*Lep*) in the absence or presence of D-APV, MK-801, DXM, or ML204 (10 μM). The *gray bar* denotes time used to quantify the mean change in fluorescence. The *inset* shows responses to bath application of 50 μM NMDA. *E*, group data for baseline-normalized changes in fluorescence for each condition. *Colored bars* correspond to representative Ca^{2+} transients shown in *D*. Data are shown as means (*bars*) along with individual cell responses (*open circles*). *Error bars* represent S.E. *, $p < 0.05$. *Veh*, vehicle.

sorted into specific secretory vesicles from the trans-Golgi network, and/or are they endocytosed together for subsequent recycling to the membrane? Knowing how Kv2.1 and K_{ATP} channels are trafficked will undoubtedly provide new avenues for drug development to overcome diseases related to insulin and leptin misregulation.

There is increasing evidence that insulin secretion from β-cells is regulated by glutamate (34). In addition to expressing NMDARs and other glutamate receptors (13–15), pancreatic β-cells express vesicular glutamate transporters VGLUT1 and VGLUT3 (35), the excitatory amino acid transporter EAAT2 (36), and the glial glutamate transporter GLT1 (37), suggesting active regulation of intracellular and extracellular glutamate signals. Several possible physiological sources of glutamate for β-cells have been reported, including circulating plasma glutamate in the range of ~20–30 μM (38), which is well above the EC_{50} of ~1 μM for NMDARs (11); glutamate released by α-cells (39); glutamate released by β-cells that is not coupled to secretion (40); and finally glutamate co-released from insulin granules (36). The possibility that β-cells may co-release glutamate during insulin secretion is intriguing. Such a mechanism would allow NMDAR activation to be coordinated with periods of insulin release. Thus, NMDAR activation may provide autoinhibitory feedback to prevent the oversecretion of insulin per-

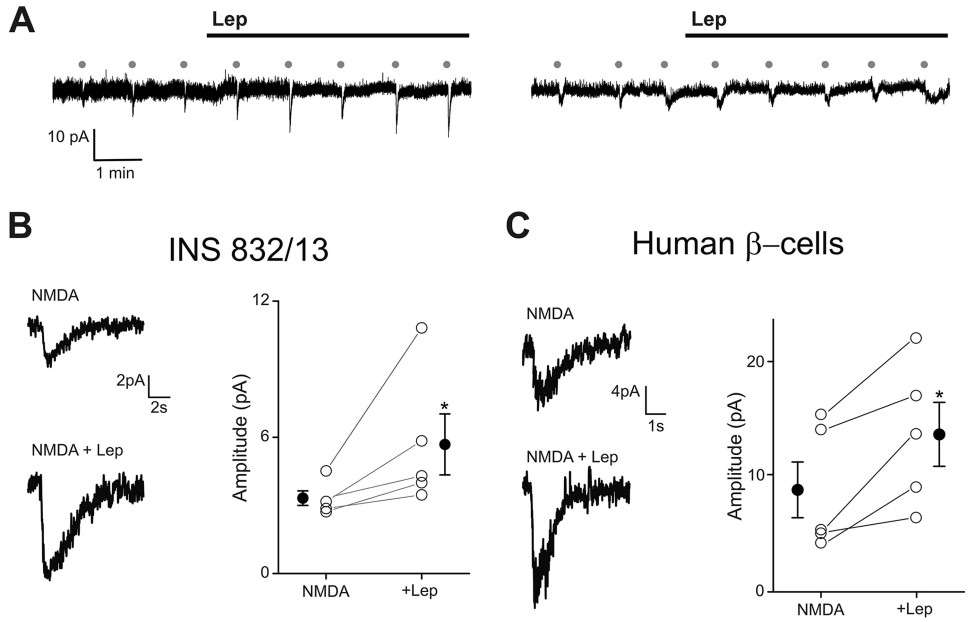

Figure 7. Leptin potentiates NMDAR currents. *A*, whole-cell NMDA current responses recorded from an INS-832/13 cell bathed in Tyrode's solution supplemented with 0.1 mM glycine but no Mg^{2+} or glucose that potentiated (*left*) or failed to potentiate (*right*) in the presence of leptin (*Lep*). Cells were recorded from a holding potential of −70 mV. *Small black dots* indicate time of NMDA puff. Group data for five INS-832/13 cells (*B*) or five human β-cells (*C*) showing the mean (*filled circles*) and individual (*open circles*) NMDA current amplitudes before and after leptin are shown. *Error bars* represent S.E. *, $p < 0.05$.

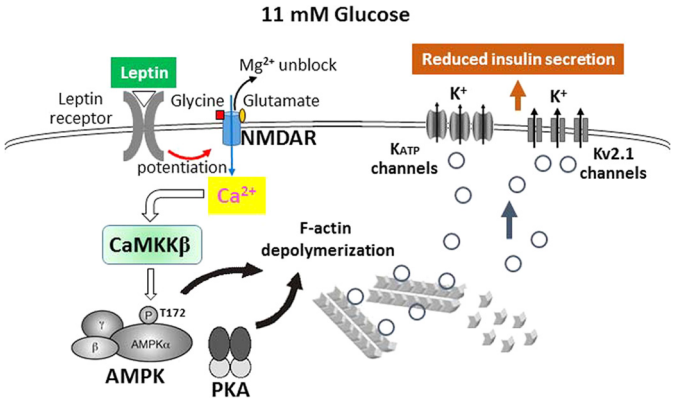

Figure 8. Proposed model depicting leptin signaling through NMDARs to regulate potassium channel trafficking in β-cells. Under 11 mM glucose, β-cells are depolarized such that NMDARs are not blocked by Mg^{2+} and are activated by glutamate and glycine present in the extracellular milieu. Binding of leptin to its receptor potentiates NMDAR currents, which leads to an increase in Ca^{2+} influx, activation of CaMKKβ, and phosphorylation of Thr-172 on AMPKα, which then cause PKA-dependent F-actin depolymerization to promote K_{ATP} and Kv2.1 channel trafficking to the plasma membrane. The increased K^+ efflux through increased surface density of Kv2.1 channels shortens action potentials and facilitates membrane repolarization, whereas the increased K^+ efflux through increased surface K_{ATP} channels leads to membrane hyperpolarization. Together, they reduce β-cell excitability and suppress insulin secretion.

haps even in the absence of leptin. In this regard, it is interesting to note that glucose stimulation has been reported to promote K_{ATP} channel trafficking to the cell surface (9).

Our results show that leptin potentiates NMDAR currents. Potentiation of NMDAR currents by leptin has been observed in other cells such as hippocampal neurons (41) and cerebellar granule cells (42). How leptin potentiates NMDARs in β-cells is not clear, although involvement of Src kinases has been implicated in hippocampal neurons (41). More studies are needed to determine whether a similar mechanism is at play in β-cells and whether these signaling molecules form a complex to confer spatial and temporal specificity.

Analysis of data from immunocytochemistry, electrophysiology, and Ca^{2+} imaging experiments revealed non-uniform NMDAR expression and responses to leptin. Studies of dispersed clonal or primary β-cells suggest that ion channel expression and composition in β-cells is heterogeneous (43, 44), including a recent report of heterogeneous NMDAR expression in the BRIN-BD11 β-cell line (24). Such cell-to-cell variations may explain controversies regarding β-cell expression of NMDARs (45, 46). Although heterogeneity among β-cells is becoming increasingly evident (47), how these subpopulations contribute to islet function and whether their expression patterns change in response to autocrine or paracrine signals are still open questions. Modeling studies have shown that heterogeneity in individual β-cell electrical properties is largely negated by the electrical coupling of β-cells via gap junctions to give rise to synchronized β-cell activity in islets (48, 49). Thus, expression of NMDARs or leptin receptors in every β-cell may not be necessary for glutamate or leptin to exert a significant impact on the overall function of an islet. In this context, NMDAR-expressing β-cells may function to trigger waves of hyperpolarization throughout the islet to suppress insulin release. Our immunocytochemistry data on dispersed human islet cells also suggest expression of NMDARs in non-β cells, although the functional significance of this finding awaits further investigation.

In summary, we demonstrate that NMDARs are unequivocally expressed in pancreatic β-cells and contribute to Ca^{2+}- and CaMKKβ-dependent trafficking of K_{ATP} and Kv2.1 channels to the plasma membrane. The signaling pathway elucidated here provides a cellular mechanism linking glutamate signaling to NMDAR-dependent regulation of insulin secretion to explain the reported antidiabetic effects of NMDAR antagonists and

Table 1
Human islet donor information
M, male; F, female; BMI, body mass index.

Donor (date received)	Age	Gender	BMI	Cause of death	Islet viability	Islet purity
					%	%
04/10/17[a]	32	M	29.2	Head trauma	97	80
04/26/17[a]	26	F	38.9	Stroke	95	95
06/30/16[b]	24	M	34.8	Head trauma	97	95
07/12/16[b]	58	M	39.3	Anoxia	95	80
07/19/16[b]	35	M	24.2	Head trauma	95	95
05/29/16[c]	33	F	34.2	Stroke	95	98
06/28/16[c]	24	M	34.8	Head trauma	99	95
07/10/16[c]	38	F	34.4	Anoxia	95	95
07/17/16[c]	35	M	24.2	Head trauma	95	95
08/02/16[d]	39	F	24.8	Stroke	90	85
08/11/16[d]	63	F	27.4	Stroke	96	85
08/24/16[d]	47	M	31.0	Anoxia	96	85
09/09/16[d]	56	M	40.1	Stroke	95	90

[a] Islets used in Fig. 1*B*.
[b] Islets used in Fig. 1*F*.
[c] Islets used in Fig. 5.
[d] Islets used in Fig. 7*C*.

further reinforces the therapeutic potential of NMDAR in the treatment of diabetes.

Experimental procedures

Cell culture

INS-1 cells (clone 832/13; referred to herein as INS-832/13) were cultured in RPMI 1640 medium with 11.1 mM D-glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (50). Human β-cells were dissociated from human islets obtained through the Integrated Islets Distribution Program as described previously (6, 10). Briefly, human islets were cultured in RPMI 1640 medium with 10% FBS and 1% L-glutamine. For recording, islets were dissociated into single cells by trituration in a solution containing 116 mM NaCl, 5.5 mM D-glucose, 3 mM EGTA, and 0.1% bovine serum albumin (BSA), pH 7.4. Dissociated cells were then plated on 0.1% gelatin-coated coverslips. For electrophysiological experiments, β-cells were identified using two separate criteria. The first criterion utilized the high autofluorescence signature of β-cells to 488-nm excitation as these cells have high concentrations of unbound flavin adenine dinucleotide (48), and the second criterion was that cells had an initial depolarizing membrane potential (~0 mV) in response to 11 mM glucose (40). Donor information for specific experiments is provided in Table 1.

Drug treatments

Leptin, tolbutamide, glutamate, and dextromethorphan were from Sigma. NMDA, (*R*,*S*)-AMPA, MK-801, D-APV, STO-609, and ML204 were from Tocris Bioscience (Bristol, UK). For surface biotinylation experiments, INS-832/13 cells were incubated in regular RPMI 1640 medium without serum for 30 min before treatment with leptin or NMDA for 30 min. Where stated, pharmacological inhibitors were added 30 min before and during the addition of leptin and NMDA.

Immunoblotting

INS-832/13 cells were lysed in lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 100 mM NaCl, 1% Triton X-100, pH 7.4, with Complete protease inhibitor) for 30 min at 4 °C, and cell lysates were cleared by centrifugation at 21,000 × *g* for 10 min at 4 °C. Proteins were separated by SDS-PAGE (7.5–12.5%) and transferred onto nitrocellulose or PVDF membranes (Millipore). Membranes were incubated overnight at 4 °C with a primary antibody diluted in TBST (Tris-buffered saline plus 0.1% Tween 20) followed by incubation with horseradish peroxidase-conjugated secondary antibodies in TBST for 1 h at room temperature. Antibodies against GluN1 and Kv2.1 (clone K89/34) were from NeuroMab (Davis, CA). Antibodies against AMPK and phosphorylated AMPK (pAMPK) were from Cell Signaling Technology (Danvers, MA) and Millipore, respectively. Antibody for SUR1 was generated in rabbit using a C-terminal peptide (KDSVFASFVRADK) of hamster SUR1 as described previously (51). Blots were developed using Super Signal West Femto (Pierce) and imaged with FluorChemE (ProteinSimple, San Jose, CA). Blots were stripped and reprobed with anti-tubulin (Sigma) as a control for loading. The blots were quantified with ImageJ (National Institutes of Health) and normalized to the corresponding controls.

Immunocytochemistry

INS-832/13 cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, permeabilized with 0.2% Triton X-100, and blocked for 60 min with 1% BSA in PBST (PBS + 0.1% Tween 20) before being incubated overnight at 4 °C with primary antibodies directed against GluN1 (NeuroMab) and insulin (Cell Signaling Technology). Proteins were visualized using Cy3- and Alexa Fluor 488-conjugated secondary antibodies. Fluorescence images were acquired using a Zeiss LSM780 confocal microscope equipped with a 63× oil immersion objective. Images were processed and analyzed using NIH ImageJ software.

Electrophysiology

Electrical recordings were performed using an Axon 200B amplifier (Molecular Devices, Sunnyvale, CA) and were filtered at 2 kHz and digitized and acquired at 20 kHz using pCLAMP software. For whole-cell patch clamp recordings, cells were held at −70 or 40 mV using micropipettes pulled from non-

heparinized Kimble glass (Thermo Fisher Scientific, Waltham, MA) filled with a solution containing 140 mM potassium gluconate, 6 mM EGTA, 10 mM HEPES, 5 mM K_2ATP, 1 mM $CaCl_2$, pH 7.2 (3–5-megaohm tip resistance). External Tyrode's solution contained 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM Na-HEPES, 3 mM $NaHCO_3$, and 0.16 mM NaH_2PO_4, pH 7.2. The external solution was supplemented with 0.1 mM glycine or 11 mM glucose, and in some experiments, Mg^{2+} was omitted as specified in the figure legends. To induce glutamate- or NMDA-mediated currents, glutamate (1 mM) or NMDA (1 mM) was puffed (3–5 p.s.i. for 0.5 s) with carbogen using micropipettes with 0.5–1-megaohm tip resistance connected to a DMF1000 Microforge multifunction controller (World Precision Instruments, Sarasota, FL) equipped with a pressure regulator.

Cell-attached recording electrodes were pulled as described above and filled with 140 mM NaCl and had tip resistances of 2–6 megaohms. The liquid junction potential was calculated to be 0 mV. Seal resistances ranged from 1 to 5 gigaohms between the recording pipette and the cell membrane. Membrane potentials were recorded in current clamp mode. Signals were analyzed using Clampfit (pCLAMP).

Surface biotinylation

INS-832/13 cells were washed twice with cold PBS and incubated with 1 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in PBS for 30 min on ice. The reaction was terminated by incubating cells for 5 min with PBS containing 50 mM glycine followed by three washes with cold PBS. Cells were then lysed in 300 μl of lysis buffer as described above, and 500 μg of total lysate was incubated with 100 μl of an ~50% slurry of NeutraAvidin-agarose beads (Pierce) overnight at 4 °C. Biotinylated proteins were eluted with 2× protein loading buffer for 15 min at room temperature. Both eluent and input samples (50 μg of total cell lysate) were analyzed by immunoblotting using anti-SUR1 or anti-Kv2.1 antibody described previously (6, 10).

Calcium imaging

INS-832/13 cells were loaded in the dark with 2 μM Fluo-4 AM (Thermo Fisher Scientific) according to the manufacturer's instructions. To prevent indicator extrusion by organic anion transporters, probenecid (2.5 mM) (Thermo Fisher Scientific) was added during loading (52). Cells were then imaged on an upright Leica microscope outfitted with a 40× water immersion objective (0.8 numerical aperture) and a Polychrome IV monochromator light source (TILL Photonics, Munich, Germany) and continuously perfused with Tyrode's solution (with 0.1 mM glycine but no Mg^{2+} or glucose) at room temperature (21–25 °C). Note that glucose was not included to avoid glucose-induced Ca^{2+} signals, which would make it difficult to discern NMDA- or leptin-evoked Ca^{2+} signals; Mg^{2+} was also not included to avoid blocking of NMDARs under no-glucose conditions. Fluo-4 fluorescence was excited at 480 nm, and fluorescence emission was filtered through a 525 (50)-nm single-band bandpass filter (Chroma Technology, Bellows Falls, VT). Images were acquired using a 12-bit ORCA-ER charge-coupled device camera (Hamamatsu, Japan) controlled by Metafluor image acquisition software (Molecular Devices). Images were acquired every 0.5 s and digitized. Fluorescence intensity was analyzed post hoc in regions of interest manually drawn around individual INS-832/13 cells using Metafluor. Changes in intracellular Ca^{2+} concentration were normalized to baseline and expressed as -fold change in $\Delta F/F_0 = ((F - F_0)/F_0)$ where F_0 is the average, background-subtracted baseline fluorescence and F is the fluorescence intensity immediately following the addition of NMDA or leptin.

Statistical analysis

Results are expressed as means ± S.E. Differences were tested using one-way analysis of variance followed by the post hoc Dunnett's test for multiple comparisons. When only two groups were compared, unpaired or paired Student's t tests were used where indicated. The level of statistical significance was set at $p < 0.05$.

Author contributions—Y. W. designed and performed experiments, analyzed data, and edited the manuscript. D. A. F. designed and performed experiments, analyzed data, and wrote the manuscript. V. A. C. designed and performed calcium imaging experiments, analyzed data, and edited the manuscript. P.-C. C. conceived the project, designed and performed experiments, and analyzed data. S.-L. S. conceived the project, designed experiments, and wrote the manuscript. All authors have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors reviewed the results and approved the final version of the manuscript.

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Note added in proof—In the version of the article that was published as a Paper in Press on August 2, 2017, Fig. 2D did not indicate the border between different sections of an immunoblot. This error has now been corrected and does not affect the results or conclusions of this work.

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