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IRS-2 Deficiency Impairs NMDA Receptor-Dependent Long-term Potentiation

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The beneficial effects of insulin and insulin-like growth factor I on cognition have been documented in humans and animal models. Conversely, obesity, hyperinsulinemia, and diabetes increase the risk for neurodegenerative disorders including Alzheimer’s disease (AD). However, the mechanisms by which insulin regulates synaptic plasticity are not well understood. Here, we report that complete disruption of insulin receptor substrate 2 (Irs2) in mice impairs long-term potentiation (LTP) of synaptic transmission in the hippocampus. Basal synaptic transmission and paired-pulse facilitation were similar between the 2 groups of mice. Induction of LTP by high-frequency conditioning tetanus did not activate postsynaptic N-methyl-D-aspartate (NMDA) receptors in hippocampal slices from Irs2−/− mice, although the expression of NR2A, NR2B, and PSD95 was equivalent to wild-type controls. Activation of Fyn, AKT, and MAPK in response to tetanus stimulation was defective in Irs2−/− mice. Interestingly, IRS2 was phosphorylated during induction of LTP in control mice, revealing a potential new component of the signaling machinery which modulates synaptic plasticity. Given that IRS2 expression is diminished in Type 2 diabetics as well as in AD patients, these data may reveal an explanation for the prevalence of cognitive decline in humans with metabolic disorders by providing a mechanistic link between insulin resistance and impaired synaptic transmission.

Keywords: diabetes, insulin receptor signaling, long-term potentiation, NMDA receptor, synaptic plasticity

Introduction

Memory deficits that develop during the course of Alzheimer’s disease (AD) have been linked to abnormalities in circulating insulin levels and/or defects in insulin signaling pathways (Luchsinger et al. 2004; Rivera et al. 2005; Craft 2007). Consistent with this, administration of insulin improves cognitive function in these patients (Kern et al. 2001; Reger et al. 2008). Evidence obtained from epidemiological studies and animal models suggest that diabetes and other metabolic disorders increase the risk for development of neurodegenerative disorders (Kuusisto et al. 1997; Vanhanen et al. 2006; Ronnemaa et al. 2008). Additionally, obesity in middle age predisposes for impaired cognitive function in the elderly (Elias et al. 2003; Whitmer et al. 2005).

Despite the accumulation of evidence to support a physiological as well as pathological role for insulin in the hippocampus, the molecular mechanisms by which this hormone modulates synaptic plasticity and memory formation remain poorly defined. Systemic insulin enters the brain through a receptor-mediated saturable transport (Bauru et al. 1993). Serum insulin-like growth factor I (IGF-I) also crosses the blood-brain barrier by a transport mechanism which is regulated directly by neuronal activity; electrical, sensory, or behavioral stimulation of neurons can increase IGF-I in activated regions (Nishijima et al. 2010). IGF-I itself has been detected in astrocytes and neurons of the central nervous system (CNS) (García-Segura et al. 1991; García-Estrada et al. 1992). The receptors for insulin (IR) and IGF-I (IGFIR) as well as other components of these signaling pathways are expressed throughout the mammalian brain with particularly high concentrations in the hypothalamus, the hippocampus, and the cerebral cortex (Raizada et al. 1988; Unger et al. 1989; Pons 1991 #392).

The cellular effects of insulin and IGF-I are mediated principally by the insulin receptor substrate (IRS) proteins (Sun et al. 1991). Upon phosphorylation by activated hormone receptors, IRS proteins recruit various signaling molecules including phosphoinositide-3-kinase (PI3K), Fyn kinase, and Grb2 (Sun et al. 1991). The deletion of IRS1 in mice reduces body size (Araki et al. 1994) and increases lifespan (Selman et al. 2008), whereas complete disruption of IRS2 causes diabetes due to insulin resistance and pancreatic β cell failure (Withers et al. 1999; Burks et al. 2000). IRS1, 2, and 4 are expressed in neurons and glia throughout the CNS including cerebral cortex, hippocampus, and hypothalamic nuclei, where they can be colocalized with the IR or IGFIR (Folli et al. 1994; Ye et al. 2002).

Learning in rodents alters the expression and phosphorylation of IR in the CA1 region of the hippocampus (Zhao et al. 1999; Dou et al. 2005). In contrast, experimental diabetes in animal models is associated with defects of synaptic transmission (Yamato et al. 2004; Ye et al. 2005). Once in the CNS, insulin modulates components of synaptic plasticity including recruitment of postsynaptic γ-aminobutyric acid (GABA) receptors (Wan et al. 1997; Vetiska et al. 2007), endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Beattie et al. 2000; Lin et al. 2000), and N-methyl-D-aspartate receptor (NMDAR) trafficking and function (Liu et al. 1995; Skeberdis et al. 2001; van der Heide et al. 2005). Recently, the IR has also been implicated in the regulation of synapse density within the Xenopus retina (Chiu et al. 2008).
One major obstacle to elucidating the precise role of insulin in cognition has been the lack of appropriate animal models; the majority of published studies have relied on the induction of diabetes in rodents by treatment with streptozocin, an antineoplastic agent which inhibits DNA synthesis and is associated with deleterious side effects including neurotoxicity (Rivera and Ajani 1998; Imaeda et al. 2002). With the present study, we have tested whether the insulin-resistant state induced by complete deletion of Irs2 in mice alters the molecular events of synaptic transmission. Here, we provide evidence that Irs2 deficiency impairs tetanus-induced long-term potentiation (LTP) in the Schaffer collateral-CA1 region.

Materials and Methods

Animals
The generation and routine genotyping of Irs2-deficient mice have been described previously (Withers et al 1998). Mice used for the present studies were maintained on a C57Bl/6 background. All experiments were performed in compliance with national regulations regarding animal welfare and were approved by the institutional committee for ethics and animal use. For routine measurements of glucose and insulin, mice were fasted for 16 h, and a small quantity of blood was obtained from the tail vein. Glucose levels were determined by glucometer (Bayer Elite). Insulin was measured by enzyme-linked immunosorbent assay (Merckod, Sweden).

Histology
Brains were fixed in 4% paraformaldehyde. Following embedding in paraffin, serial sections of 5 microns were prepared. Subsequently, sections were deparaffinized and stained using a standard Nissl protocol.

Electrophysiology
Transverse brain slices (400 µm thickness) were prepared from female mice (3 months old) as described previously (Martin and Buno 2003) and incubated for 1 h at room temperature (21–24 °C) in ACSF infused with 95% O₂ and 5% CO₂. The artificial cerebral spinal fluid (ACSF) contained (in mM): NaCl 124, KCl 2.69, KH₂PO₄ 1.25, MgSO₄ 2, NaHCO₃ 26, CaCl₂ 2 and glucose 10. Prior to use, ACSF was equilibrated when filled with the internal solution that contained (in mM) the following: cesium gluconate 107.5, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20, ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA) 0.2, NaCl 18, TEACl 10, Mg-ATP 4, and GTP 0.3 at pH 7.3 (adjusted with CsOH), with osmolarities between 280 and 290 mOsm/L. Whole-cell recordings in the voltage-clamp mode were obtained with an 2400 patch amplifier (A-M Systems). Fast and slow capacitances were neutralized; series resistance was compensated (≥80%), and membrane potential (Vm) was held at ~60 mV. Data were discarded if the series resistance changed by more than 20% during an experiment. Evoked EPSPs were elicited by stimulation of Schaeffer collateral fibers as described previously for the fEPSPs recording. Data were filtered at 2 KHz and transferred to the hard disk of a Pentium-based computer using a DigiData 1440A interface and the pCLAMP 10.0 software (Axon Instruments, Molecular Devices Corporation, Sunnyvale, CA). For whole-cell recordings, LTP was induced either by tetanic stimulation in current clamp mode or pairing postsynaptic depolarization to 0 mV with stimulation of presynaptic Schaeffer collateral fibers at 2 Hz during 60 s (Martin and Buno 2003). To isolate EPSPs from GABA₂-mediated inhibitory synaptic transmission, experiments were performed in the presence of 50 µM bicuculline. With these conditions, we isolated AMPA and NMDA EPSP components using an experimental approach based on differential voltage dependence and kinetics (Collingridge et al. 1983; Hestrin et al. 1993; Hestrin et al. 1990; Martin and Pozo 2004). Indeed, when EPSPs are recorded in the presence of extracellular Mg²⁺, the AMPA and NMDA EPSP can be isolated because at ~60 mV, only the fast AMPA EPSP is recorded due to the voltage-dependent block of the NMDA receptor channel by extracellular Mg²⁺, while at +60 mV, the slower NMDA receptor-mediated EPSP is present due to the Mg²⁺ block relief of the NMDA receptor channels (Collingridge et al. 1983; Hestrin et al. 1993; Martin and Pozo 2004). At +60 mV and at delays >50 ms, the AMPA EPSP has completely disappeared, whereas the slower NMDA EPSP is peaking and its amplitude can be estimated in isolation (Hestrin et al. 1990; Martin and Pozo 2004). All drugs were purchased from Sigma (St Louis, MO) except CNQX, APS, and bicuculline that were from Tocris Cookson (Bristol, UK). Statistical differences were established using the 2-tailed Student’s t-test.

Immunoprecipitation and Western Blotting
Hippocampus slices were prepared for electrophysiology as described above. Prior to the induction of LTP, slices were collected and processed as nonstimulated controls. Following the induction of LTP, hippocampal slices were collected at 5 and 30 min and frozen immediately in liquid N₂. Tissue was lysed in RIPA buffer by polytron, and homogenates were clarified by centrifugation at 12 000 g for 10 min. Protein determination was by the Bradford assay. Immunoprecipitations were performed using 500 µg of total protein. The immune complexes were collected on protein A-agarose or protein G-agarose beads and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to Immobilon membranes and incubated with one of the following antibodies: anti-IRB2 (Upstate), anti-phosphotyrosine (Upstate), anti-p-AKT (Cell Signaling), anti-MAPK (Santa Cruz Biotechnology Inc.), anti-IR (Santa Cruz Biotechnology Inc.), anti-IGFIR (Cell Signaling). Westerns were developed using the ECL (Pierce), films scanned and immunoreactive bands quantified with the Alpha Imager program.

Isolation of Postsynaptic Densities
Mice were sacrificed by cervical dislocation, and the hippocampus was dissected from each brain. The fixed angle protocol of Villasana et al. (2006) was adapted for tablettop ultracentrifuge (Beckman Coulter Optima TLX-120) using 4 hippocampi of each genotype. Briefly, hippocampi were pooled and lysed by homogenization in ice-cold buffer (320 mM sucrose, 4 mM HEPES, pH 7.3) to which a cocktail of inhibitors was added (Complete, Roche). An aliquot of the homogenate was collected for western analysis of total lysate (T) and the remainder...
was centrifuged for 10 min at 1000 x g in Eppendorf tubes. The resulting pellet was discarded and the supernatant was centrifuged 9200 x g for 15 min. The pellet containing crude synaptosomes was resuspended in hypotonic lysis buffer and centrifuged for 20 min at 250 000 x g. The pellet containing the postsynaptic densities (PSD) was solubilized (50 mM Tris, 150 mM NaCl, 2.5 mM ethylenediaminetetraacetic acid, 2.5 mM EGTA, pH 7.4, containing protease inhibitors), and protein concentration was measured by Bradford assay. Forty micrograms of protein from the total lysate, cytosolic fraction, and PSD fraction were separated by SDS-PAGE, and western analysis was performed as described above.

Results

**Basal Synaptic Transmission Is Preserved in Irs2^{−/−} Mice**

We first analyzed basal synaptic transmission by applying isolated stimuli of increasing intensity to the Schaffer collaterals (Fig. 1A). Input/output curves for extracellular fEPSP were indistinguishable between slices from Irs2^{−/−} mice and wild-type (WT) controls. For a range of stimulation intensities, the slopes of Irs2^{−/−} fEPSP responses were not significantly different from the fEPSP responses of WT slices (P > 0.05, n = 6 mice of each genotype, at least 3 slices per mouse, Fig. 1B). Likewise, measurements of the fiber volleys amplitude from Irs2^{−/−} and WT slices were similar (P > 0.05, n = 6 mice of each genotype, at least 3 slices per mouse, Fig. 1C) and there was no difference in an input/output curve (Fig. 1D). We also investigated presynaptic function to exclude the possibility that the absence of IRS2 alters the probability of neurotransmitter release. PPF ratios of fEPSP slopes at interstimulus intervals ranging from 25 to 400 ms were normal in the Irs2^{−/−} mice (P > 0.05, n = 6 mice of each genotype, at least 3 slices per mouse, Fig. 1E,F). Collectively, these results indicate that Irs-2 deficiency does not modify the basal synaptic transmission at the presynaptic or postsynaptic level.

**Loss of IRS2 Function Disables LTP at the Postsynaptic Level**

Given that insulin resistance is associated with impaired neuronal function, we used the Irs2 transgenic model to assess synaptic plasticity at Schaffer collateral--CA1 synapse in the hippocampus using a high-frequency conditioning tetanus to induce LTP. LTP is the sustained increase in synaptic strength obtained after a high-frequency conditioning stimulus and is a compelling model for the synaptic mechanism underlying some forms of learning and memory (Malenka and Nicoll 1999). Baseline responses were monitored for 10–30 min before conditioning and were found to be stable. Tetanic conditioning revealed a marked difference in the ability of hippocampus slices from Irs2^{−/−} mice to support LTP, with potentiation of fEPSP being significantly reduced in slices from transgenic animals as compared with WT (n = 6 mice of each genotype, at least 3 slices per mouse, P < 0.001, Fig. 2A). To evaluate whether the lack of tetanus-induced LTP might reflect insufficient cumulative depolarization during tetanic stimulation, LTP was induced by pairing postsynaptic depolarization with presynaptic stimulation via patch-clamp techniques in whole-cell recordings (see Materials and Methods). This LTP was also completely impaired in Irs2^{−/−} mice (n = 4 mice of each genotype, at least 2 slices per mouse, P > 0.001, Fig. 2B), indicating that IRS2 signaling is required to maintain synaptic plasticity at Schaffer collateral--CA1 synapse in the hippocampus. To test the possibility that increased inhibition causes the impaired LTP in Irs2^{−/−} mice, tetanic conditioning was applied to control and transgenic slices in the presence the GABA_A receptor antagonist bicuculline (50 μM). However, under these conditions, the defective LTP persisted in Irs2^{−/−} mice (Fig. 2C), demonstrating that increased inhibition was not responsible for the inability to induce LTP in the absence of IRS2 signals. Moreover, PTP of the fEPSP revealed no significant differences between slices from mutant and control animals (mean percent of baseline before tetanus: Irs2^{−/−} 98.7 ± 1.2%, n = 6 mice; Irs2^{−/−} 99.6 ± 0.8%, n = 6 mice; following tetanus: Irs2^{−/−} 256.1 ± 15.4%, n = 6 mice; Irs2^{−/−} 217.2 ± 21.1%, n = 6 mice). PTP is postulated to indicate presynaptic function, reflecting a period of enhanced transmitter release caused by the loading of the presynaptic terminals with calcium ions during tetanic conditioning (Zucker 1989). Therefore, these results demonstrate clearly that the impairment of LTP observed in the hippocampus of Irs2^{−/−} is due to defects at the postsynaptic level without a reduction of neurotransmitter release. Moreover, the impaired LTP was not related with diabetic complications in the Irs2^{−/−} animals since 8-week-old females with normal glucose and insulin levels were...
used for all experiments of the current study (Supplementary Fig. 1A,B). Deletion of Irs-2 produces diabetes in mice due to reduced beta cell mass and peripheral insulin resistance; however, the development of diabetes in this model displays a sexual dimorphism which affects males earlier and more severely than females (Burks et al. 2000). Given the critical role of IRS-2 in brain development (Schubert et al. 2003), we examined the formation of the hippocampus but found no gross morphological differences between genotypes (Supplementary Fig. 1C).

**Irs2−/− Mice Display an Inadequate Synaptic Activation of NMDA Receptors**

LTP in the hippocampus is expressed as an increase of the slope and amplitude of the fast component of EPSP mediated by the AMPA subtype of glutamate receptor (Schubert et al. 2003). However, it is generally agreed that induction of the classical LTP requires the activation of postsynaptic NMDA receptors (Bliss and Collingridge 1993). Consistent with this, the NMDAR antagonist AP5 completely abolished the induction of LTP in WT and Irs2−/− mice (n = 4 mice of each genotype; at least 2 slices per mouse, Fig. 3A), confirming that this type of LTP indeed requires NMDA receptor activation. To quantify the relative contributions of AMPA- and NMDA receptor-mediated synaptic components under our experimental conditions, patch-clamp recordings from whole cells were made during the first 30 min following tetanic stimulation under current clamp mode, using an experimental approach based on different voltage dependence and kinetics (see Materials and Methods). The AMPA receptor-mediated component of the evoked EPSC (AMPA<sub>EPSC</sub>) measured at −60 mV increased 5 min after tetanic stimulation in WT and Irs2−/− samples (Fig. 3B,C). However, although the AMPA<sub>EPSC</sub> amplitude was maintained 30 min after tetanus in WT (Fig. 3B,C), these recordings returned...
to baseline values in Irs2\(^{-/-}\) animals. The amplitude of NMDAR-mediated component (NMDA\(_{\text{EPSC}}\)), measured at +60 mV and at delays of 100 ms, was not significantly different from baseline values at 5 and 30 min after tetanus in WT slices (\(n = 4\) mice of each genotype, at least 3 slices per mouse, Fig. 3B,D), in agreement with previous observations (Kullmann 1994; Nicoll and Malenka 1999; Poncer and Malinow 2001). This potentiation of the AMPA\(_{\text{EPSC}}\) component, without changes to the NMDA\(_{\text{EPSC}}\) component, characterizes the classical LTD of Schaeffer collateral EPSCs in CA1 pyramidal neurons (Kullmann 1994; Nicoll and Malenka 1999; Poncer and Malinow 2001). However, with slices from Irs2\(^{-/-}\) mice, the NMDA\(_{\text{EPSC}}\) declined significantly to below baseline values 30 min after tetanus (63 ± 21%, \(n = 4\) mice of each genotype, at least 3 slices per mouse, \(P < 0.05\), Fig. 3B,D). The observed deficit of the NMDAR-mediated component of EPSC in Irs2\(^{-/-}\) slices could be the result of either alterations to the number or properties of the synaptic NMDA receptors or inadequate activation of NMDA receptors during the high-frequency stimulation. To distinguish between these possibilities, we first examined the ratio of NMDAR-mediated to AMPA receptor-mediated synaptic currents, which controls for slice-to-slice variability in the number of synapses activated (Saal et al. 2003). First, the AMPA receptor-mediated synaptic current amplitude was measured at a holding potential of -60 mV. Then, recording from the same cell, the NMDAR-mediated current was recorded at a holding potential of +60 mV and its amplitude was measured at delays >50 ms (see Materials and Methods). The calculated ratio was similar between Irs2\(^{-/-}\) and WT mice (Fig. 3B,E). We next investigated whether synaptic activation of AMPA or NMDA receptors was normal using fEPSP recordings during a stimuli train (100 Hz) where the NMDA component was isolated with CNQX in Mg\(^{2+}\)-free solution and the AMPA component with AP5 in normal ACSF (Hestrin et al. 1990; Martin and Pozo 2004). Under these conditions, the recordings for the AMPA receptor-mediated component of fEPSP were similar between WT and Irs2\(^{-/-}\) slices (Fig. 4A,B). In contrast, we observed that the percentage of the second, third, and fourth fEPSP slope over the first fEPSP in the train of isolated NMDA component was significantly smaller in the Irs2\(^{-/-}\) when compared with that of WT slices (second fEPSP: 137.5 ± 19.6% for Irs2\(^{-/-}\) vs. 59.6 ± 19.5% for Irs2\(^{+/+}\), \(P < 0.05\); third fEPSP: 45.3 ± 15.7% for Irs2\(^{-/-}\) vs. 7.2 ± 12.1% for Irs2\(^{+/+}\), \(P < 0.01\); 35.3 ± 11.2% for Irs2\(^{-/-}\) to fourth fEPSP, \(P < 0.01\); \(n = 4\) mice of each genotype, at least 3 slices per mouse, Fig. 4C,D). Collectively, these results demonstrate that Irs2 null mice display inadequate activation of postsynaptic NMDARs.

**Tyrosine Phosphorylation of NR2B Subunit of NMDA Receptors Is Reduced in Irs2 Null Mice**

We next performed a series of experiments to elucidate the contributions of NMDAR subtypes to the defect observed in Irs2 null mice. We recorded pharmacologically isolated NMDAR-mediated EPSCs by using whole-cell voltage clamp in the presence of 50 μM bicuculline and 20 μM CNQX in Mg\(^{2+}\)-free external solution. Application of ifenprodil (3 μM), an NR2B-subtype-specific antagonist, reduced the NMDAR-mediated EPSCs in WT mice (Fig. 5A; 51.8 ± 7%; \(n = 4\) mice of each genotype, at least 2 slices per mouse). In contrast, the same treatment had only a minor effect on NMDAR-mediated EPSCs of Irs2 null mice (Fig. 5A; 91.9 ± 9%; \(n = 4\) mice of each genotype, at least 2 slices per mouse). These results strongly suggest that NR2B-containing NMDARs play an important role in the synaptic plasticity modulated by IRS2 signaling.

Various lines of evidence indicate that the NR2B subunit of NMDA receptors undergoes tyrosine phosphorylation during

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**Figure 4.** Inadequate activation of synaptic NMDA receptors during the high-frequency stimulation in the Irs2\(^{-/-}\) transgenic mice. (A) Representative field responses of AMPA component evoked by a 100 Hz train during 200 ms in Irs2\(^{+/+}\) (top traces) and Irs2\(^{-/-}\) (bottom traces), recorded in the presence of 50 μM AP5 to block NMDA component. (B) Summary data showing mean AMPA isolated fEPSP slopes in Irs2\(^{+/+}\) (filled bar; \(n = 4\)) and Irs2\(^{-/-}\) (open bar; \(n = 4\)) mice, that were plotted against the number of the stimulus during 100 Hz train. The fEPSP slope at the 4 initial stimuli was calculated as the percentage of the first fEPSP slope. (C) Representative field responses of NMDA component evoked by a 100-Hz train during 200 ms in Irs2\(^{+/+}\) (top tracings) and Irs2\(^{-/-}\) (bottom tracings), recorded in the presence of 20 μM CNQX to block AMPA component. (D) Summary data showing mean NMDA isolated fEPSP slopes in Irs2\(^{+/+}\) (filled bar; \(n = 4\)) and Irs2\(^{-/-}\) (open bar; \(n = 4\)) mice. The fEPSP slope was significantly smaller in the Irs2\(^{-/-}\) for the second, third, and fourth stimuli. Significant differences were established at \({}^*P < 0.05\) and \({}^{**}P < 0.01\).
induction of LTP (Rostas et al. 1996). The NR2B contains 3 tyrosine phosphorylation sites which are targeted by Fyn (Nakazawa et al. 2001). To assess the tyrosine phosphorylation of NR2B in IRS-2 deficient mice, hippocampal slices were collected prior to induction of LTP and at 5 and 30 min after tetanic stimulation. These slices were then lysed and analyzed by western blotting. Consistent with previous reports (Rostas et al. 1996), tetanus increased significantly the tyrosine phosphorylation of NR2B after 5 min in WT slices (Fig. 5B), and this activation persisted until 30 min. However, in hippocampus slices from Irs2–/– mice, tetanus stimulation did not produce tyrosine phosphorylation of NR2B at either of the time points (Fig. 5B).

The failure to trigger tyrosine phosphorylation of NR2B with tetanus stimulation could not be attributed to reduced expression of this subunit since the levels detected by western blotting in hippocampus slices were equivalent between WT and Irs2 mutant mice (Fig. 5B,C). Given that NR2B appeared to be inactive in Irs2–/– mice, we assessed whether increased expression of NR2A might reflect a compensatory mechanism in this model. However, western blotting analysis of hippocampus lysates revealed that the expression of NR2A (as well as NR2B) was equivalent between both genotypes (Fig. 5C). Another potential explanation for the impaired function of NR2B is failed targeting to the PSD in the hippocampus of Irs2 KO animals. The PSD is a structural network of receptors, ion channels, and signaling proteins which are required for synaptic function (Feng and Zhang 2009). To investigate this possibility, PSD fractions were prepared from hippocampus of both experimental groups. Aliquots of total lysate, cytosol, and PSD were analyzed by western blotting. PSD95, a scaffolding protein which forms complexes with NMDAR (Sheng 2001), was used as a marker of PSD. Due to the significant enrichment of PSD95 in the PSD fraction, a longer exposure (40 s) was required to detect these proteins in the total lysate as illustrated by the additional image of PSD95.
Irs2 Deficiency Impairs Activation of Signaling Pathways Associated with LTP and Synaptic Transmission

To relate the impaired LTP to a molecular mechanism, we next examined intracellular signal transduction during the induction of LTP. Upon activation of insulin and IGF-I receptors by ligand binding, these receptor tyrosine kinases phosphorylate IRS proteins, enabling them to recruit and activate other signaling molecules (White 1997). Fyn is a nonreceptor tyrosine kinase which mediates the effects of insulin in various tissues (Davidson et al. 1994; Mastick and Saltiel 1997). Interestingly, ablation of Fyn in mice impairs LTP without alterations to basal synaptic transmission and PPF (Grant et al. 1992). Upon insulin stimulation, Fyn associates with phosphorylated tyrosine residues of IRS proteins via its src homology (SH) 2 domain (Sun et al. 1996). Therefore, we evaluated the activation of Fyn in response to tetanus stimulation in hippocampus slices from WT and Irs2-deficient mice. At 5 min poststimulus, the activation of Fyn as detected by anti-phosphotyrosine antibodies was increased significantly in WT slices (Fig. 6A) and persisted at 30 min, although the levels were slightly lower than at 5 min. In sharp contrast, tetanus stimulation did not induce tyrosine phosphorylation of Fyn in Irs2−/− slices at 5 min; however, after 30 min, the levels of tyrosine-phosphorylated Fyn were comparable to those detected in WT slices. Thus, these results suggest that IRS2 signals are required for the proper temporal phosphorylation of Fyn in response to high trains of tetanus stimulation.

Once phosphorylated on specific tyrosine residues, IRS proteins also recruit and activate PI3-K through a direct interaction with the p85 catalytic subunit (Myers et al. 1992). Interestingly, PI3K activity and its subsequent phosphorylation of Akt are also necessary for expression of LTP (Man et al. 2003; Wang et al. 2003). In our analysis of signal transduction pathways, we observed that the levels of phosphorylated Akt increased 5 min after the induction of LTP in slices from WT mice (Fig. 6B) and returned to basal levels after 30 min. However, in hippocampus slices from Irs2−/− mice, tetanus did not produce an increase of phospho-Akt at 5 min, perhaps owing to the fact that the basal levels of phospho-Akt were higher than in slices from WT controls (Fig. 6B). In peripheral tissues such as liver and muscle, Irs2-deficiency has been associated with elevated basal levels of PI3K and AKT which impairs the ability of insulin or IGF-I to activate these pathways (Withers et al. 1998).

The MAPK/ERK pathway mediates certain forms of NMDA-dependent LTP (English and Sweatt 1996; Winder et al. 1999; Kanterewicz et al. 2000; Shalin et al. 2006). Insulin can activate MAPK/ERK either directly via its receptor or by the IRS/Grb2/ ras complex (Avruch 1998). Analysis of ERK phosphorylation in
hippocampus slices revealed that at 5 min poststimulus there were no differences between WT and Irs2−/− mice; however, after 30 min, phospho-ERK levels remained elevated in WT samples (Fig. 6C), whereas they returned to basal levels in Irs2−/− slices. Thus, these results demonstrate that IRS-2 signals are essential for sustained activation of ERK during the induction of LTP by tetanic stimulation.

The defects in these signaling pathways could not be explained by alterations to the expression of the IR, IGF-IR, or IRS-1 since the levels of these proteins in hippocampus were equivalent between WT and Irs2−/− mice (Fig. 6D). Given that the ability of IRS proteins to orchestrate intracellular signaling is dependent on their tyrosine phosphorylation, we next examined the possibility that induction of LTP by high trains of tetanus stimulation promotes the tyrosine phosphorylation of IRS2. Thus, IRS2 was immunoprecipitated from WT hippocampus slices which had been subjected to tetanus stimulation, and the immune complexes were analyzed subsequently by western blotting using anti-phosphotyrosine antibodies. Interestingly, the tyrosine phosphorylation of IRS2 was increased significantly at 5 min poststimulus but returned to basal levels after 30 min (Fig. 6E). Collectively, these results are consistent with a role for IRS2 in recruiting and activating other signaling pathways implicated in the expression of LTP including Fyn kinase and Akt.

**Discussion**

Previous studies have demonstrated that IRS2 is required for neuronal development (Schubert et al. 2003) and for CNS-mediated regulation of appetite (Burks et al. 2000). Whole-body deletion of Irs2 causes insulin resistance by disabling insulin signaling pathways; Irs2-deficiency causes hyperphagia and obesity due to impaired insulin signaling in specific populations of hypothalamic neurons (Kubota et al. 2004; Choudhury et al. 2005). In the present study, we have identified IRS2 as a novel component of the signaling apparatus which mediates synaptic plasticity in the hippocampus. Through a series of electrophysiological studies, we have demonstrated that expression of LTP is significantly impaired in Irs2 null mice. This impairment of LTP occurs at the postsynaptic level without a reduction to neurotransmitter release since we did not detect significant differences in paired-pulse facilitation, synaptic fatigue, or PTP between WT and Irs2−/− mice.

Several observations from our study demonstrate that IRS2 signals may participate in the activation of postsynaptic NMDA receptors during high-frequency stimulation. First, Irs2−/− mice did not present alterations to the number or properties of the synaptic NMDARs. Second, when we isolated specifically the synaptic NMDA population of LTP, we observed a significant decline to below baseline values 30 min after tetanus in Irs2−/− mice, while no changes were noted in WT control animals. Finally, the EPSPs slopes of the isolated NMDA component in a 100 Hz train (the frequency that induces LTP under our experimental conditions) were significantly smaller in the Irs2−/− slices when compared with those registered in control slices. Importantly, the ratio of NMDA/AMPA synaptic currents, which controls for slice-to-slice variability in the number of synapses activated (Saal et al. 2003), was similar between Irs2−/− and control mice. Modulation of synaptic transmission appears to be a specific role for IRS-2 since mice deficient for IRS1, 3, or 4 have no reported CNS defects (Araki et al. 1994; Liu et al. 1999).

The present observation that IRS2 signals participate directly in LTP raises questions about precisely how this docking molecule modulates synaptic plasticity. Our analysis of the signaling pathways during induction of LTP in hippocampus slices has revealed that the absence of IRS2 impairs tyrosine phosphorylation of NR2B and activation of Fyn kinase, AKT, and MAPK in hippocampal slices. Interestingly, insulin and IGF-I regulate these pathways by SH2 domains (Schlessinger et al. 1992). By employing IRS proteins to engage other signaling molecules, the IR circumvents stoichiometric constraints encountered by receptors that directly recruit SH2 proteins to their autophosphorylation sites; one IRS molecule can simultaneously bind various SH2-containing molecules (e.g., Fyn, p85 PI3K, and Grb2). Given that IRS proteins are not tethered to the plasma membrane, the recruitment of these molecules to IRS2 may serve the critical function of relocating activated signaling pathways to a site within the neuron where they can modulate synaptic plasticity.

Mice deficient for Fyn kinase display impaired LTP and deficits of spatial learning (Grant et al. 1992). However, no studies to date have addressed the upstream signals that activate Fyn during the induction of LTP. We observed that Fyn was fully phosphorylated 5 min after the application of tetanus trains in normal mice, whereas this did not occur until 30 min in hippocampus slices from Irs2−/− mice. Given that Fyn is activated upon the interaction of its SH2 domain with phosphorylated IRS proteins (Sun et al. 1996), our findings suggest that IRS2 plays an important role in regulating the activity of this kinase. Thus, when IRS2 signals are absent or reduced, the delayed postsynaptic activation of Fyn may impair the ability of this kinase to phosphorylate the NR2B subunits of NMDA receptors, an event which potentiates NMDA channel activity (Yu et al. 1997; Xu et al. 2006). Our studies demonstrate that the expression of NR2A and NR2B as well as their targeting to PSDs is preserved in Irs2-deficient mice, excluding these potential alterations as explanations for the impaired LTP.

Irs2 deficiency has been shown previously to impair the activation of PI3K/AKT in liver and muscle of Irs2−/− mice (Withers et al. 1998). PI3K has been linked to the insertion of AMPA receptors at activated CA1 synapses (Man et al. 2003) and to the inhibition of GSK3β activity during the induction of LTP (Peineau et al. 2007), whereas AKT activity is required for the cell surface expression of GABA receptors (Wang et al. 2003). Hence, our observation that conditioning tetanus is unable to activate AKT in hippocampus slices of Irs2-deficient mice suggests that IRS2 serves as an important upstream element for the regulation of synaptic PI3K/AKT signaling.

Surprisingly, induction of LTP in WT hippocampus slices increased tyrosine phosphorylation of IRS2. Given that IRS proteins are phosphorylated exclusively by receptors for insulin and IGF-I, this observation requires further study to determine precisely how IRS2 becomes activated during application of conditioning tetanus to hippocampus slices. Depolarization of rat neuronal cultures by potassium ions has been reported to stimulate a significant release of insulin (Clarke et al. 1986). Therefore, it is possible that induction of LTP in the Schaeffer collateral fibers by tetanus trains causes neurons and/or astrocytes to release insulin and/or IGF-I into their surroundings and this, in turn, induces the phosphorylation of IRS2 via the activation of postsynaptic insulin receptors. Alternatively, physiological levels of systemic insulin may modulate synaptic plasticity in vivo by promoting the
phosphorylation of IRS2 within neurons that are receptive to LTP. Whole-body deletion of Irs2 causes insulin resistance and other metabolic abnormalities by disabling insulin signaling pathways; Irs2-deficient mice eat more than control mice due to the presence of insulin resistance in specific populations of hypothalamic neurons (Burks et al. 2000; Kubota et al. 2004; Chodbury et al. 2005). Similarly, failed LTP in the hippocampus of Irs2 null mice may also reflect a form of insulin resistance which undermines the proper activation of AKT and Fyn in the PSD.

Studies of human subjects as well as experimental animal models demonstrate that diabetes adversely affects learning and memory (Biessels et al. 1996; Desrocher and Rovet 2004; Messier 2005; Stranahan et al. 2008). Our present results suggest that IRS2 expression and/or function may represent an important link between metabolic disorders and synaptic plasticity. Irs2 expression is significantly reduced in pancreatic islets from humans with Type 2 diabetes (Gunton et al. 2005), consistent with a critical role for IRS2 in maintaining normal sensitivity to insulin. Interestingly, reduced expression and/or function of insulin and IGF-I signaling molecules have been detected in AD brains (Craft 2007; Moloney et al. 2008). By establishing a molecular connection between systemic insulin/IGF-I resistance and impaired synaptic transmission, our observations suggest that lifestyle changes or pharmacological agents that target the expression and/or function of IRS2 may prevent neuronal dysfunction in patients with metabolic diseases.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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