Claudia A. Grillo,[1] Gerardo G. Piroli,[1] Robert C. Lawrence,[1,2] Shayna A. Wrighten,[1] Adrienne J. Green,[1] Steven P. Wilson,[1] Randall R. Sakai,[3] Sandra J. Kelly,[1,2] Marlene A. Wilson,[1,4] David D. Mott,[1] and Lawrence P. Reagan[1,4]

Hippocampal Insulin Resistance Impairs Spatial Learning and Synaptic Plasticity

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Insulin receptors (IRs) are expressed in discrete neuronal populations in the central nervous system, including the hippocampus. To elucidate the functional role of hippocampal IRs independent of metabolic function, we generated a model of hippocampal-specific insulin resistance using a lentiviral vector expressing an IR antisense sequence (LV-IRAS). LV-IRAS effectively downregulates IR expression in the rat hippocampus without affecting body weight, adiposity, or peripheral glucose homeostasis. Nevertheless, hippocampal neuroplasticity was impaired in LV-IRAS–treated rats. High-frequency stimulation, which evoked robust long-term potentiation (LTP) in brain slices from LV control rats, failed to evoke LTP in LV-IRAS–treated rats. GluN2B subunit levels, as well as the basal level of phosphorylation of GluA1, were reduced in the hippocampus of LV-IRAS rats. Moreover, these deficits in synaptic transmission were associated with impairments in spatial learning. We suggest that alterations in the expression and phosphorylation of glutamate receptor subunits underlie the alterations in LTP and that these changes are responsible for the impairment in hippocampal-dependent learning. Importantly, these learning deficits are strikingly similar to the impairments in complex task performance observed in patients with diabetes, which strengthens the hypothesis that hippocampal insulin resistance is a key mediator of cognitive deficits independent of glycemic control.

Metabolic syndrome and diabetes are disorders of the brain as much as of the body, yet it is unclear whether specific impairments in brain or systemic insulin receptor (IR) signaling are primarily responsible for neurological consequences of diabetes, including cognitive dysfunction. IRs are expressed in discrete neuronal populations in the central nervous system, including the hippocampus (1). Since the hippocampus is a critical integration center for learning and memory, the hippocampal IRs are proposed to facilitate cognitive function (2). Clinical studies have demonstrated that type 2 diabetes mellitus (T2DM) affects hippocampal volume and impairs hippocampal-based memory performance (3,4) and that intranasal insulin improves cognitive function in the absence of metabolic changes (5–7). These data support the idea that the cognitive-enhancing properties of insulin may be mediated through IRs expressed in the hippocampus. This relationship between IR activity and behavioral performance has also been examined in experimental models of T2DM (8,9). Similar observations were reported in a mouse model of Alzheimer disease (AD) (10). These data suggest that central insulin resistance is a key mechanistic factor for diabetes-mediated neuroplasticity deficits. Since clinical and epidemiological data also illustrate that patients with diabetes have an increased risk of developing age-related cognitive disorders like AD (11), these data also support the hypothesis that deficits in IR signaling may be a crucial initiating factor in the development and progression of AD-related cognitive decline.

In order to more specifically study the role of IRs on cognitive function, we developed a lentiviral vector that contains a selective IR antisense sequence (LV-IRAS) (12). When injected in the third ventricle to target IRs expressed in the arcuate nucleus, IR expression and signaling are significantly decreased in the hypothalamus compared with rats treated with a control virus (LV-Con). Hypothalamic downregulation of IRs results in increased body weight, greater subcutaneous adiposity, and increased plasma leptin levels. These changes are consistent with the proposed role of hypothalamic IRs

[1]Department of Pharmacology, Physiology & Neuroscience, University of South Carolina School of Medicine, Columbia, SC
[2]Department of Psychology, University of South Carolina, Columbia, SC
[3]Department of Psychiatry, University of Cincinnati Medical Center, Cincinnati, OH
[4]William Jennings Bryan Dorn Veterans Affairs Medical Center, Columbia, SC
Corresponding author: Claudia A. Grillo, cgrillo@uscmed.sc.edu.

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in the regulation of body weight and metabolism and are similar to observations in neuronal IR knockout mice (NIRKO mice) (13) and rats treated with IR antisense oligodeoxynucleotides (14). In the current study, we have used the LV-IRAS construct to selectively downregulate IRs in the hippocampus to develop a model of hippocampal-specific insulin resistance to examine the mechanistic basis of insulin in neuroplasticity. This model allows us to test the hypothesis that IR signaling plays a key role in hippocampal plasticity and cognitive function, independent of any metabolic changes. Accordingly, the goals of the current studies were to examine the mechanistic role of IRs in hippocampal synaptic plasticity (long-term potentiation [LTP]), hippocampal-dependent behavioral tasks such as Morris water maze (MWM), and glutamatergic receptor subunit expression proposed to be involved in hippocampal synaptic plasticity.

RESEARCH DESIGN AND METHODS

Animal Protocols

Two-month-old male Sprague Dawley rats (CD strain; Charles River) weighing 200–250 g were housed in groups of three with ad libitum access to food and water. All procedures involving animals were carried out in accordance with guidelines and regulations of the University of South Carolina Animal Care and Use Committee. Lentivirus production and administration were performed as described in our previous studies (12,15,16). In brief, rats were anesthetized and placed in the stereotaxic apparatus, and lentivirus was injected into the hippocampus at the following coordinates: 4.8 mm posterior to bregma, 4.0 mm lateral to midline, and 3.0 mm ventral to dura. Five microliters of the viral stock (5×10^6 TU/μL) was injected at a speed of 0.2 μL/min for 25 min with a 10-μL Hamilton syringe driven by a motorized stereotaxic injector (Stoelting 53310); the needle was left in place for an additional 15 min. Rats were injected with either LV-IRAS or the LV-Con construct; each rat received bilateral injections of the same viral construct. After surgery, LV-IRAS and LV-Con rats were housed individually in a BSL2 facility for at least 3 weeks prior to subsequent analyses. All experiments were performed when the rats were <4 months of age.

Immunohistochemical Approaches

In order to analyze the distribution of the lentivirus infused into the hippocampus, immunohistochemistry for the reporter gene green fluorescent protein (GFP) was performed as described in our previous studies (12,17). In brief, rat brain sections were washed with PBS and then incubated with primary antisera raised against GFP (1:1,000 dilution; Sigma-Aldrich, St. Louis, MO). After an overnight incubation at 4°C, sections were washed in PBS and then incubated in biotinylated donkey anti-mouse secondary antibody (1:600) for 90 min at room temperature. After washes with PBS, sections were incubated with horseradish peroxidase–conjugated streptavidin (1:100) at room temperature for 1 h. GFP immunoreactivity was developed using diaminobenzidine as a substrate.

Plasma Endocrine Analysis

To determine changes in metabolic parameters, LV-treated rats were subjected to an oral glucose tolerance test (OGTT) as described in our previous studies (12,15). A cohort of rats was subjected to an overnight fast, and the following morning a blood sample was collected for baseline measures. Glucose (2 g/kg) was then administered by gastric intubation. Blood samples were collected from the tip of the tail at the following time points after intubation: 30, 60, 90, and 120 min. Blood glucose levels were measured by the glucose oxidase method (Pointe Scientific, Inc., Canton, MI); plasma insulin levels were measured by ELISA (Millipore, Billerica, MA).

To determine stress responses, a separate cohort of LV-treated rats were subjected to an acute restraint stress as described in our previous studies (16). Plasma corticosterone (CORT) levels were determined by ELISA (Enzo, Farmingdale, NY). Plasma leptin levels were also determined by ELISA (Millipore) in LV-Con and LV-IRAS rats (12,15). ELISA plates were analyzed according to the manufacturer's instructions using a Tecan SPECTRAFluor plate reader (Tecan U.S., Inc., Durham, NC). Statistical analysis was performed using a two-tailed, unpaired Student t test with $P < 0.05$ as the criterion for statistical significance.

Immunoblot Analysis

Immunoblotting analysis was performed in a separate cohort of rats as described in our previous studies (12,17,18). In brief, membrane fractions were separated by SDS-PAGE (10%), transferred to polyvinylidene difluoride (PVDF) membranes, and blocked in Tris-buffered saline (TBS) plus 10% nonfat dry milk for 60 min. PVDF membranes were incubated with primary antisera in TBS/5% nonfat dry milk. After overnight incubation at 4°C, blots were washed with TBS plus 0.05% Tween 20 (TBST) and incubated with peroxidase-labeled species-specific secondary antibodies. PVDF membranes were then washed with TBST and developed using enhanced chemiluminescence reagents (Amersham) as described by the manufacturer. Normalization for protein loading was performed using a mouse monoclonal primary antibody selective for actin.

In Vitro Phosphorylation Assays

In vitro phosphorylation of the IRs and Akt was performed as described in our previous study (12) based upon protocols developed by Alkon and coworkers (19). In brief, 50 μg protein of hippocampal total membrane fractions was incubated with reaction buffer (50 mmol/L Tris-HCl, pH 7.4; 1 mmol/L $MgCl_2$; 2 mmol/L EGTA; 1× protease inhibitor cocktail [Sigma-Aldrich]; 1× phosphatase inhibitor cocktail [Sigma-Aldrich]). In vitro phosphorylation was stimulated by the addition of 1 μmol/L insulin and 5 mmol/L ATP. After addition of insulin/ATP, samples were incubated for 3 min at 37°C. SDS-PAGE sample

buffer was quickly added; the samples were boiled for 10 min and loaded into a precast 4–20% SDS-PAGE gel (Bio-Rad).

Autoradiographic Analysis of Immunoblots

Computer-assisted microdensitometry of autoradiographic images was determined on the MCID image analysis system (Imaging Research, Inc., St. Catharines, Canada), as previously described (12,15). Gray level/optical density calibrations were performed using a calibrated film strip ladder (Imaging Research, Inc.) for optical density. Statistical analysis was performed using a two-tailed, unpaired Student *t* test with $P < 0.05$ as the criterion for statistical significance.

MWM

Using a separate cohort of animals, LV-Con and LV-IRAS rats were subjected to behavioral testing for spatial memory in the MWM, ~4 weeks after virus administration. The maze consisted of a black circular pool (200 cm in diameter) filled with water (temperature 23 ± 1°C, depth 40 cm) made opaque with nontoxic tempura paint situated in a room with visual cues on the walls. A black platform (10 cm in diameter) was submerged in the water (2 cm below the water surface), and the pool was conceptually divided into four quadrants and had four points designed as starting positions: N, S, W, or E (20).

Reference Memory Protocol

In this task, rats received 4 training days (sessions) and a probe trial in the 5th day. Each session consisted of four trials with a 5-min intertrial interval. A trial began when the rat was placed in the water at one of the four starting positions, chosen at random, facing the wall. The order of the starting position varied in every trial, and any given sequence was not repeated on acquisition phase days. The rat was given 60 s to locate the platform; if the animal did not succeed, it was gently guided to the platform and left on it for 15 s. Rats were dried and returned to their home cages after each trial. The latency to find the platform was measured in each trial, and the mean latency for every training day was calculated. The probe consisted of a single trial, with the platform removed, and was performed 1 h after the last trial. Data were collected using the EthoVision (Noldus, Leesburg, VA) automated system. Statistical analysis on the dependent variables MWM performance was performed by two-way repeated-measures ANOVA, with treatment and time as independent variables and sessions as the repeated measure. All analyses were followed by a Bonferroni post hoc test, with $P < 0.05$ as the criterion for statistical significance.

Hippocampal Slice Electrophysiology

Using a separate cohort of animals, transverse brain slices (500 μm thick) were prepared from isoflurane-anesthetized rats in sucrose-based "cutting" artificial cerebrospinal fluid, and field excitatory postsynaptic potentials (fEPSPs) were recorded from stratum radiatum of CA1b as previously described (16). Baseline test stimuli were delivered every 30 s at a stimulus intensity that evoked a fEPSP that was 30% of maximal slope. After at least 10 min of stable baseline responses, LTP was induced with an high frequency stimulus (HFS) train (100 Hz, 1 s) delivered at the test stimulus intensity. Statistical analysis was performed using a two-way ANOVA followed by a Bonferroni post hoc test, with $P < 0.05$ as the criterion for statistical significance.

Statistics

Values are reported as mean ± SEM. All statistical analysis was performed in GraphPad Prism 6 software. Simple comparisons were analyzed using a two-tailed, unpaired Student *t* test. For multiple comparisons, two-way ANOVA followed by Bonferroni post hoc test was applied since the data were normally distributed. Significance was set at $P < 0.05$.

RESULTS

Spreading of the Lentivirus in the Hippocampus

In order to analyze the dissemination of the lentivirus infused into the rat hippocampus, we performed immunohistochemistry for GFP, the reporter gene included in the lentivirus construct. GFP was effectively expressed throughout the whole hippocampus, including the Ammon's horn and the dentate gyrus (DG), and was not expressed in the adjacent areas such as the cortex (Fig. 1*A*). Conversely, GFP immunoreactivity was not observed in the hypothalamus of rats that received intrahippocampal injection of the lentivirus (Fig. 1*B*), confirming that the lentivirus spreading is limited to the hippocampus. The GFP was expressed in the CA3 (Fig. 1*C*), CA1 (Fig. 1*D*), and DG (Fig. 1*E*), demonstrating the ability of the lentivirus to reach all subfields of the hippocampus.

Downregulation of Hippocampal IR After LV-IRAS Administration

Our previous studies demonstrate that LV-IRAS successfully downregulates IR expression and activity in the rat hypothalamus (15). In order to determine the downregulation of IRs in the hippocampus, we performed Western blot analysis in hippocampal plasma membrane fractions. LV-IRAS rats exhibited significant decreases in hippocampal IR expression compared with LV-Con rats. Autoradiographic analysis determined that intrahippocampal LV-IRAS administration reduced IR expression by 70% (Fig. 2*A*). In order to determine the effects of LV-IRAS upon IR function in the hippocampus, insulin-stimulated phosphorylation of the IRs, the first step in the insulin signaling, was analyzed. Incubation of hippocampal total membrane fractions with insulin and ATP produced a robust increase in the phosphorylation state of the IRs. Autoradiographic analysis revealed that in vitro phosphorylation of the IRs was significantly reduced by 70% in the hippocampus of LV-IRAS rats compared with LV-Con rats 3 weeks after intrahippocampal lentivirus administration (Fig. 2*B*). This reduction in insulin-stimulated

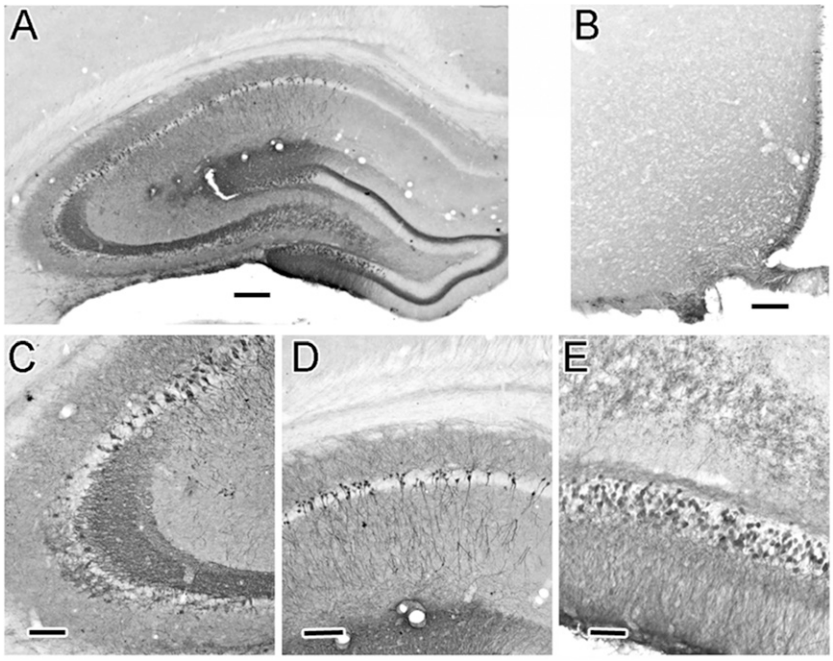

Figure 1—GFP is expressed in the Ammon's horn and DG of rats infused with lentivirus. *A*: Representative immunohistochemical (IHC) labeling for GFP shows a consistent expression of the reporter protein across all the hippocampus subfields, without labeling in adjacent areas such as the cortex. *B*: Representative IHC labeling for GFP in the hypothalamus exhibits lack of expression in this area. *C*: Representative IHC labeling for GFP in the CA3 subfield of the hippocampus. *D*: Representative IHC labeling for GFP in the CA1 subfield of the hippocampus. *E*: Representative IHC labeling for GFP in the DG subfield of the hippocampus. Scale bar, 200 μm for panel *A* and 100 μm for panels *B–E*.

phosphorylation of hippocampal IRs was comparable to the levels achieved when targeting hypothalamic IRs (12,15–17). In order to further determine the effects of the LV-IRAS construct upon IR function in the hippocampus, insulin-stimulated phosphorylation of Akt, a key step in the insulin signaling, was analyzed using the same in vitro phosphorylation protocol described above. Incubation of hippocampal total membrane fractions with insulin and ATP produced a robust increase in the phosphorylation of Akt. Autoradiographic analysis revealed that in vitro phosphorylation of Akt was significantly reduced in the hippocampus of LV-IRAS rats compared with LV-Con rats (Fig. 2*C*).

Whereas lentivirus-mediated downregulation of hypothalamic IRs increases body weight, body adiposity, and plasma leptin levels (12,15), downregulation of hippocampal IRs had no effect upon these parameters (Fig. 3*A–C*). Downregulation of hippocampal IRs did not affect glucose clearance or insulin release in response to an OGTT (Fig. 3*D* and *E*). Additionally, acute restraint stress–induced increases in plasma CORT levels did not differ between the groups, indicating that stress reactivity is not affected by downregulation of hippocampal IRs (Fig. 3*F*). These data confirm the efficacy of LV-IRAS to downregulate hippocampal IRs and elicit hippocampal insulin resistance. Moreover, these results further emphasize the region-specific activities of hypothalamic and hippocampal IRs.

Downregulation of Hippocampal IRs Impairs Hippocampal-Dependent Learning

In order to evaluate spatial memory in our model of hippocampal insulin resistance, performance in the MWM was evaluated in LV-Con and LV-IRAS rats. Two-way ANOVA on latencies revealed significant main effects of downregulation of hippocampal IRs ($F_{(1,56)}$ = 11.09, P = 0.0015) and a significant effect of time ($F_{(3,56)}$ = 10.95, $P < 0.0001$), with no significant interaction ($F_{(3,56)}$ = 0.79, P = 0.5064) on the latency to find the platform (Fig. 4*A*). An analysis of separate days was conducted to examine whether there were any differences in performance in separate trials. During the first day, both groups of rats behaved in an identical fashion (Fig. 4*B*). However, after this first day, LV-IRAS rats showed impairments in their ability to find the platform compared with the control rats, especially on the first trial of each day. In the second session, two-way ANOVA revealed a significant effect of downregulation of hippocampal IRs ($F_{(1,56)}$ = 12.62, P = 0.0007), an effect of trials ($F_{(3,56)}$ = 10.62, $P < 0.0001$), and a significant interaction ($F_{(3,56)}$ = 4.22, P = 0.0088) on the latency to find the platform (Fig. 4*C*). In the third session, two-way ANOVA revealed a significant effect of downregulation of hippocampal IRs ($F_{(1,56)}$ = 12.88, P = 0.0007) and an effect of trials ($F_{(3,56)}$ = 10.13, $P <$ 0.0001), but no significant interaction ($F_{(3,56)}$ = 2.05, P = 0.1172) on the latency to find the platform (Fig. 4*D*). In the fourth session, two-way ANOVA revealed

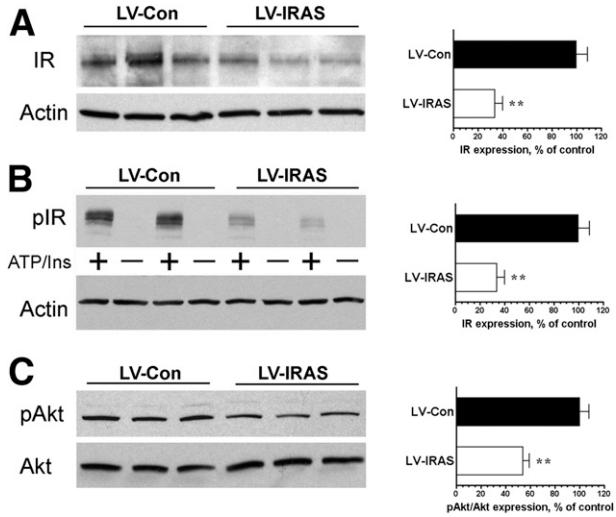

Figure 2—IR expression and insulin-stimulated phosphorylation of the IR and Akt are decreased in the hippocampus of LV-IRAS–treated rats. *A*: Representative immunoblot of IR levels in the hippocampus of LV-Con– and LV-IRAS–treated rats. Normalization for protein loading was performed using a monoclonal antibody for actin. Autoradiographic analysis determined that IR levels are significantly decreased in the hippocampus of the LV-IRAS–treated rats compared with LV-Con–treated rats. Data, representing mean ± SEM (n = 8 per group), were analyzed by Student t test (**P < 0.01). *B*: Representative immunoblot of phosphorylated IR (pIR) levels in the hippocampus of LV-Con– and LV-IRAS–treated rats. Normalization for protein loading was performed using a monoclonal antibody for actin. Autoradiographic analysis determined that pIR levels are significantly decreased in the hippocampus of the LV-IRAS–treated rats compared with LV-Con–treated rats. Data, representing mean ± SEM (n = 8 per group), were analyzed by Student t test (**P < 0.01). +, Insulin/ATP treatment; −, buffer. *C*: Representative immunoblot of phosphorylated Akt (pAkt) levels in the hippocampus of LV-Con– and LV-IRAS–treated rats. Normalization for protein loading was performed using an antibody for Akt. Autoradiographic analysis determined that the pAkt-to-Akt ratio is significantly decreased in the hippocampus of the LV-IRAS–treated rats compared with LV-Con–treated rats. Data, representing mean ± SEM (n = 8 per group), were analyzed by Student t test (**P < 0.01). Ins, insulin.

a significant effect of trials ($F_{(3,56)}$ = 9.67, $P < 0.0001$), but no significant effect of downregulation of hippocampal IRs ($F_{(1,56)}$ = 1.02, P = 0.3168) or interaction ($F_{(3,56)}$ = 1.76, P = 0.1654) on the latency to find the platform (Fig. 4*E*). Post hoc Bonferroni analysis showed significant differences in the first trial of session two and three on the latency to find the platform in the LV-IRAS rats compared with the LV-Con rats ($P < 0.0001$ and $P < 0.001$, respectively). These behavioral deficits were not associated with locomotor deficits since swimming velocity did not differ between the groups (Fig. 4*F*). Probe trial analysis showed significant increases in the latency to swim to the original platform position in the rats with downregulation of hippocampal IRs compared with the LV-Con rats when they were tested 1 h after the training (Fig. 4*G*). Accordingly, the LV-IRAS rats exhibit a longer path to find the original platform position in the 1-h probe trial (Fig. 4*H*). Taken together, these results suggest that whereas short-term memory is unaffected, long-term memory is impaired in the rats with hippocampal insulin resistance.

Downregulation of Hippocampal IRs Reduces Synaptic Plasticity

Since hippocampal synaptic plasticity is adversely affected in diabetes, we examined stimulus-induced LTP in the hippocampus of LV-Con and LV-IRAS rats. Downregulation of hippocampal IRs did not impair basal synaptic transmission; the input-output curve of the fEPSP slope was not altered in LV-IRAS rats compared with LV-Con rats, suggesting no gross alteration in synaptic transmission (Fig. 5*A*). In these same rats, paired pulse facilitation of fEPSP slope was also similar to controls, suggesting that presynaptic function was not altered by downregulation of hippocampal IRs (Fig. 5*B*). In LV-Con rats, HFS of the Schaffer collaterals elicited LTP of fEPSPs in the CA1 region (Fig. 5*C*). Conversely, HFS of the Schaffer collaterals elicited short-term potentiation that failed to develop into LTP in the CA1 region of LV-IRAS rats (Fig. 5*C*). Furthermore, downregulation of hippocampal IRs blocked LTP of fEPSP not only in the CA1 region but also in the DG (Fig. 5*D*).

Downregulation of Hippocampal IRs Reduces Glutamate Receptor Subunit Expression and Phosphorylation

In view of the important role of the glutamatergic system in the facilitation of hippocampal synaptic transmission and hippocampal-dependent behaviors, we examined the expression and basal phosphorylation state of glutamate receptor subunits in the hippocampus of LV-Con and LV-IRAS rats. Hippocampal-specific deficiency in IR signaling decreases the basal phosphorylation of GluA1 at serine 845 (Fig. 6*A*), as indicated by a significant reduction in the ratio of phospho-Ser-845 GluA1 to total GluA1 in hippocampal extracts from LV-IRAS rats. In addition, IR downregulation also reduced the expression of GluN2B (Fig. 6*B*) in hippocampal extracts isolated from LV-IRAS rats, while not affecting GluA2, GluN1, and GluN2A levels (data not shown).

DISCUSSION

The results of this study demonstrate that hippocampal insulin resistance in adult normal rats reduces molecular, cellular, electrophysiological, and behavioral measures of hippocampal neuroplasticity, deficits that were observed in the absence of metabolic or endocrine imbalance. Although these alterations are identical to those observed in experimental models of T2DM, rats with hippocampal insulin resistance do not exhibit the hallmark metabolic and endocrine features of diabetic rats. Collectively, these results support the hypothesis that insulin directly facilitates hippocampal neuroplasticity, independently of the metabolic state.

The neuroprotective role of insulin has been reported in different models of T2DM and AD. For instance, defective insulin signaling is a characteristic feature of the

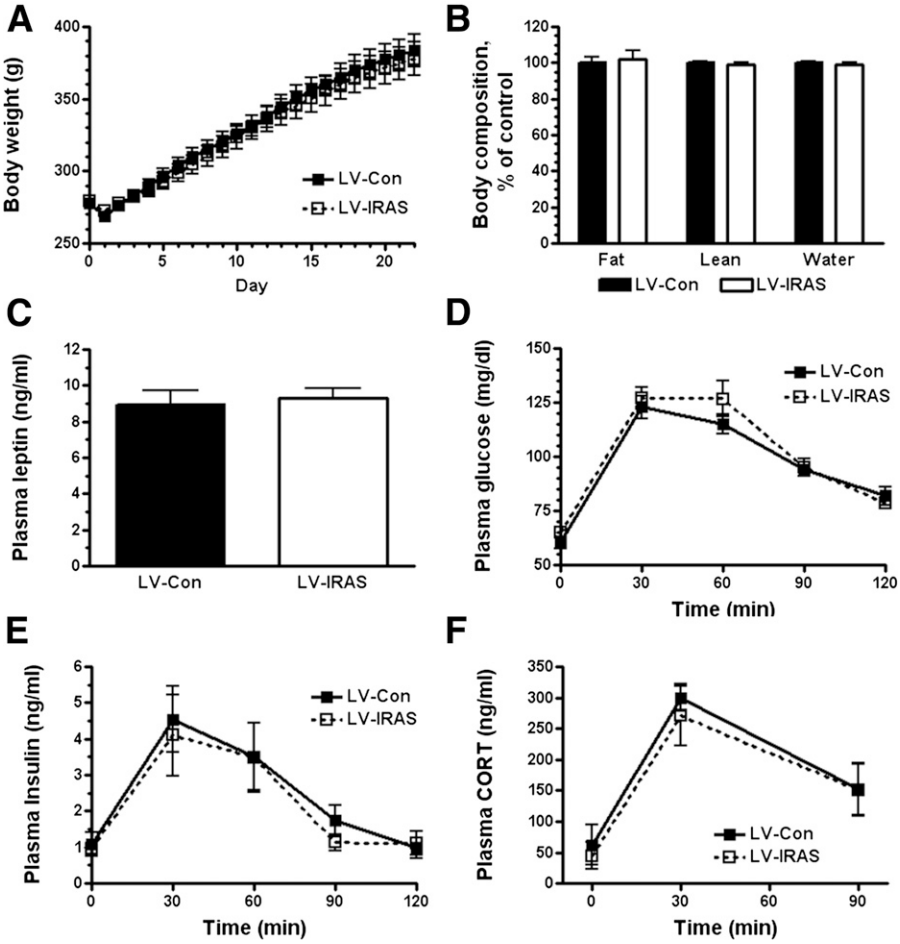

Figure 3—Physiological and endocrine parameters are not affected by downregulation of the hippocampal IRs. *A*: LV-IRAS rats did not exhibit changes in body weight compared with LV-Con rats (n = 10 per group). *B*: LV-IRAS rats do not exhibit changes in body fat, lean mass, or water content compared with LV-Con rats (n = 10 per group). *C*: Plasma leptin levels did not differ between LV-IRAS and LV-Con rats (n = 10 per group). *D*: In response to an OGTT, LV-IRAS rats exhibited similar profiles in plasma glucose (n = 6 per group). *E*: Plasma insulin levels were similarly stimulated after the OGTT in both groups. Areas under the curve results, for glucose (*D*) and insulin (*E*), were also similar between the groups (n = 6 per group). *F*: Basal, stress-induced increase and poststress levels of plasma CORT do not differ between the groups (n = 4 per group).

AD brain (21). In this regard, several studies have suggested a potential role of oligomeric amyloid-β to induce brain insulin resistance that is associated with memory impairment (22,23). In addition, intranasal insulin facilitates cognition in adults with early-stage AD (24). These results suggest that defective insulin signaling contributes to AD pathogenesis, as previously proposed. Indeed, insulin resistance is a crucial contributor to the adverse effects on hippocampal cognitive function (25). In T2DM models, the neuroplasticity deficits in the hippocampus include decreases in neuronal spine density (26), decreases in synaptic transmission (26,27), and increases in oxidative stress (28). Ultimately, the long-term consequences of diabetes-induced neuroplasticity deficits are reflected in cognitive impairments (9). Even though clinical studies in AD and diabetes support the hypothesis that insulin facilitates hippocampal neuroplasticity, these pathological conditions are accompanied by numerous metabolic and endocrine alterations that can negatively impact hippocampal integrity.

In view of the complex metabolic and endocrine milieu that is characteristic of T2DM, molecular approaches attempted to clarify the role of IRs in the hippocampal synaptic plasticity. For example, Nisticò et al. (29) reported that mice with haploinsufficiency of IR β-subunit showed reduced hippocampal LTP and deficits in recognition memory. However, these mice also exhibit metabolic disturbances, which may contribute to the hippocampal plasticity changes. Compensatory changes during development could explain the absence of behavioral deficits previously reported in NIRKO mice (30). Lentivirus-mediated gene expression avoids these developmental and metabolic limitations. Our previous studies showed that LV-IRAS was highly expressed 21 days postinjection in adult rats (12), leading to downregulation of IRs that was maintained for several months, providing an adequate time

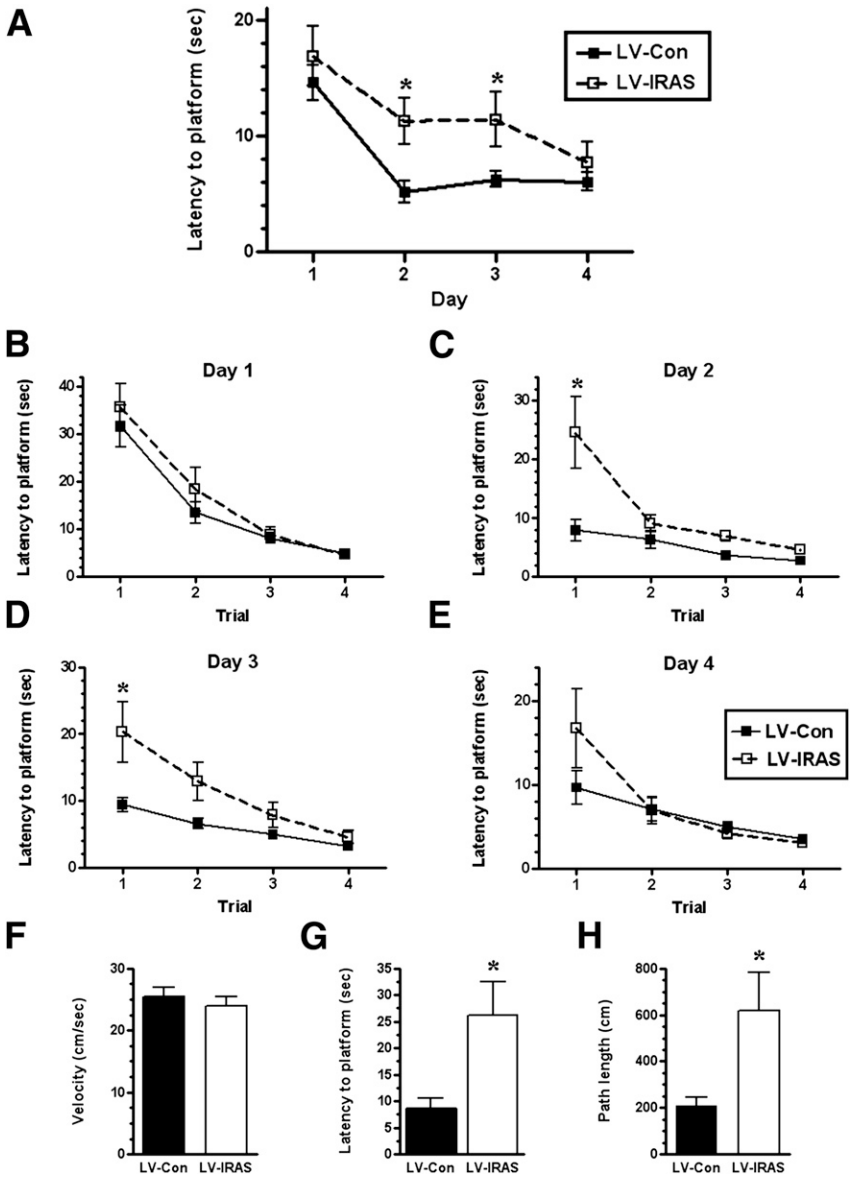

Figure 4—Performance of rats with hippocampal IR deficiency (LV-IRAS) and control rats (LV-Con) in the water maze reference memory task. *A*: Downregulation of hippocampal IRs increases the time needed to reach the hidden platform in the second and third session. Data, representing mean ± SEM (n = 9 per group), were analyzed by two-way ANOVA followed by Bonferroni post hoc test, showing that there were significant time and treatment effects (*$P < 0.05$). *B–E*: Daily performance of rats with hippocampal IR deficiency (LV-IRAS) and control rats (LV-Con) in the water maze reference memory task. Downregulation of the hippocampal IRs increased the time needed to reach the hidden platform in the first trial of the second and third sessions compared with the control group. Data, representing mean ± SEM (n = 9 per group), were analyzed by two-way ANOVA followed by a Bonferroni post hoc test (*$P < 0.05$). *F–H*: Probe trial of the water maze reference memory task. Downregulation of the hippocampal IRs (LV-IRAS) does not affect the swimming velocity compared with LV-IRAS rats (*F*). However, LV-IRAS rats needed more time (*G*) and exhibited longer paths (*H*) to reach the position where the platform was placed 1 h after the training compared with LV-Con rats. Data, representing mean ± SEM (n = 9 per group), were analyzed by Student t test (*$P < 0.05$).

frame for behavioral testing. In addition, this technique allowed us to knock down hypothalamic IRs without affecting hippocampal IR expression (12,15,17). In the current study, lentivirus-mediated downregulation of hippocampal IRs is not associated with metabolic or endocrine changes. Although it is likely that insulin resistance occurs via disruption of second messenger signaling, downregulation of hippocampal IRs provides an effective strategy to more selectively examine the functional role of the IRs on hippocampal plasticity.

Insulin, Learning, and Memory

Clinical and preclinical studies supported the hypothesis that activation of IRs improves cognition. Insulin enhances cognitive performance in healthy (5) and aged subjects (31) and even in patients with AD. Additionally, chronic

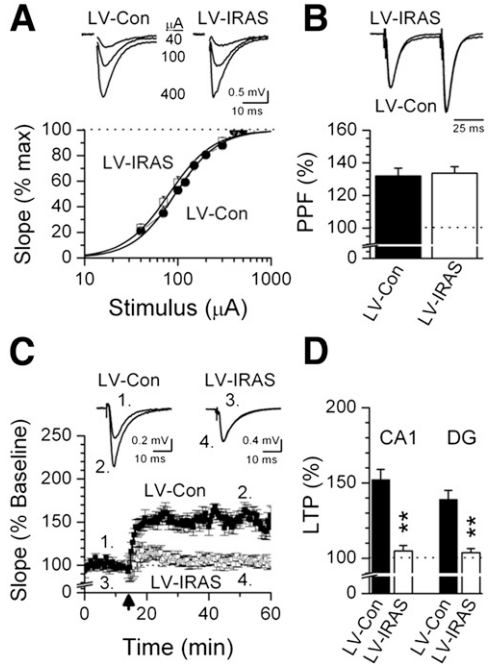

Figure 5—Downregulation of hippocampal IRs impairs LTP. Electrophysiological analysis was performed 21 days after administration of LV-Con or LV-IRAS in the CA1 region of the hippocampus. *A*: The input-output relation shows a similar fEPSP slope in both LV-Con–treated (n = 9) and LV-IRAS–treated (n = 7) animals over a range of stimulus intensities. *B*: Paired pulse facilitation (PPF) at a 50-ms interstimulus interval was similar in animals treated with LV-Con (n = 10) and LV-IRAS (n = 7). *A* and *B*, top: Superimposed fEPSPs from LV-Con and LV-IRAS animals were scaled to the amplitude of the first fEPSP to show the similarity in PPF. *C*: LV-Con–treated rats developed LTP in response to HFS (100 Hz, 1 s) of the Schaffer collaterals (arrow). In contrast, LTP, but not short-term potentiation, was blocked in LV-IRAS–treated rats. Inset shows sample fEPSPs collected at the times indicated on the graph. *D*: LV-IRAS treatment blocked LTP of the fEPSP in both CA1 (LV-Con treated, n = 7; LV-IRAS treated, n = 7; $^{**}P < 0.01$) and DG (LV-Con treated, n = 6; LV-IRAS treated, n = 4; $^{**}P < 0.01$).

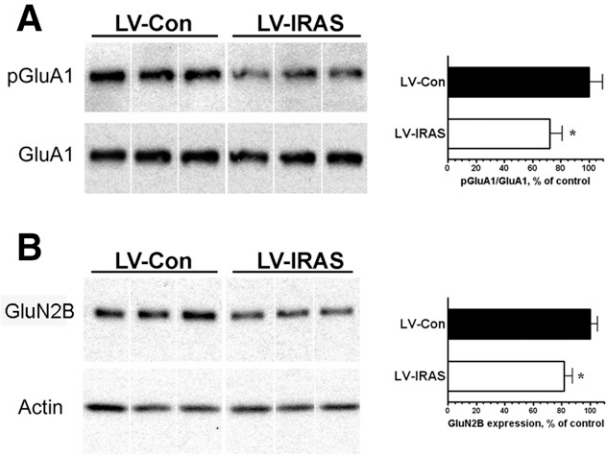

Figure 6—*A*: Phosphorylation of GluA1 subunit is decreased in the hippocampus of LV-IRAS–treated rats. Representative immunoblot of phosphorylated and total GluA1 levels in the hippocampus of LV-Con– and LV-IRAS–treated rats. Autoradiographic analysis determined that the pGluA1-to-GluA1 ratio is significantly decreased in the hippocampus of the LV-IRAS–treated rats compared with LV-Con–treated rats. Data, representing mean ± SEM (n = 10 per group), were analyzed by Student t test ($^{*}P < 0.05$). *B*: GluN2B subunit expression is decreased in the hippocampus of LV-IRAS–treated rats. Representative immunoblot of GluN2B in the hippocampus of LV-Con– and LV-IRAS–treated rats. Normalization for protein loading was performed using a monoclonal antibody for actin. Autoradiographic analysis determined that expression of GluN2B is significantly decreased in the hippocampus of the LV-IRAS–treated rats compared with LV-Con–treated rats. Each lane represents hippocampal membrane fractions from individual rats that were run on the same gel at the same time. Data, representing mean ± SEM (n = 10 per group), were analyzed by Student t test ($^{*}P < 0.05$).

intranasal insulin administration improves cognitive function in both patients with AD and nondemented patients (5,7).

Conversely, we observed that LV-IRAS rats showed impaired memory in the first trial on days 2 and 3 of the MWM testing, which is consistent with a deficit in long-term memory. However, there is not any effect across days, suggesting that working memory is not impaired in the LV-IRAS rats. These findings are consistent with the previous literature. For example, intracerebroventricular injection of insulin improves spatial memory in a dose-dependent fashion in male rats (32), whereas intra-CA1 insulin microinjections enhances behavioral performance in the water maze (33), and acute delivery of insulin into the rat hippocampus promotes spatial memory in the alternation test (34) and transiently enhances hippocampal-dependent memory in the inhibitory avoidance test (35). Conversely, behavioral deficits are consistently observed in experimental models of diabetes (9,26,27,34,36), which supports a crucial role for hippocampal IRs in the spatial learning and memory.

Insulin, Synaptic Transmission, and Glutamate Receptors

IRs are enriched at hippocampal synapses, where they are proposed to regulate synaptic plasticity through interactions with the glutamatergic system, and insulin signaling may affect synaptic plasticity by regulating glutamate receptor expression and trafficking. For example, AMPA and NMDA receptor expression and trafficking are adversely affected in the hippocampus of diabetic rodents (37). Conversely, insulin enhances NMDA-mediated synaptic transmission (38), promotes surface expression of these receptors (39), and stimulates the phosphorylation of GluN2A and GluN2B subunits in the hippocampus (40). Recent studies provided additional evidence for functional interactions between insulin and the glutamate system. IR substrate 2 (IRS-2) deficiency leads to NMDA receptor dysfunction and reduced tyrosine phosphorylation of GluN2B subunits after LTP induction (41). In addition, brain deficiency of IRS-2 had a strong impact on NMDA receptor–dependent synaptic transmission and plasticity in the CA1, which was associated with decreases in phosphorylation of GluN1 subunits (42). In the current study, we found that selective knockdown of

hippocampal IRs decreased serine 845 phosphorylation of GluA1 and expression of GluN2B, which suggests that these changes underlie the effects of insulin on LTP.

Insulin promotes extrasynaptic membrane trafficking of GluA1 AMPA receptors in cultured neurons (43), and this trafficking is regulated by serine 845 phosphorylation (44). Extrasynaptic GluA1 is proposed to regulate LTP by forming a reserve pool of "primed receptors" that can rapidly be incorporated into synapses upon NMDA receptor stimulation to enhance synaptic strength (45). On the basis of our study, we propose that insulin regulates the phosphorylation of serine 845 of GluA1 and consequently the basal level of extrasynaptic membrane trafficking of GluA1. Insulin signaling also increases expression of GluN2B subunits. These GluN2B-containing NMDA receptors are proposed to promote LTP by increasing calcium entry during synaptic activation (46) and to stabilize LTP through interactions with CaMKII (47). Downregulation of hippocampal IRs could therefore suppress LTP by reducing the following: *1*) basal phosphorylation of GluA1, thereby reducing the pool of reserve extrasynaptic receptors (44); *2*) calcium entry through GluN2B during synaptic stimulation; and *3*) LTP stability. In support of these suggestions, decreases in serine 845 phosphorylation of GluA1 correspondingly decrease its surface expression and the extent of LTP evoked by theta burst stimulation (44). Conversely, transgenic overexpression of GluN2B enhances hippocampal LTP (48). In the context of other studies that have examined the critical role of GluA1 and GluN2B subunits in synaptic plasticity and learning (48,49), our data suggest that insulin is required for the appropriate basal phosphorylation and extrasynaptic membrane trafficking of GluA1 and to maintain expression of GluN2B subunits and through these mechanisms promotes learning and memory. Additional studies are needed to confirm these possibilities.

Summary

Our results begin to disentangle the differential neurological consequences of hippocampal insulin resistance and the peripheral metabolic abnormalities that are associated with diabetes and obesity phenotypes. In addition, our model of hippocampal-specific insulin resistance supports the hypothesis that insulin per se plays a significant role in hippocampal function that is independent of glucose levels and other metabolic consequences associated with hyperglycemia.

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