Leptin Enhances NMDA Receptor Function and Modulates Hippocampal Synaptic Plasticity

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The obese gene product leptin is an important signaling protein that regulates food intake and body weight via activation of the hypothalamic leptin receptor (Ob-Rb; Jacob et al., 1997). However, there is growing evidence that Ob-Rb is also expressed in CNS regions, not directly associated with energy homeostasis (Mercer et al., 1996; Hakansson et al., 1998). In the hippocampus, an area of the brain involved in learning and memory, we have found that leptin facilitates the induction of synaptic plasticity. Leptin converts short-term potentiation of synaptic transmission induced by primed burst stimulation of the Schaffer collateral commissural pathway into long-term potentiation. The mechanism underlying this effect involves facilitation of NMDA receptor function because leptin rapidly enhances NMDA-induced increases in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and facilitates NMDA, but not AMPA, receptor-mediated synaptic transmission. The signaling mechanism underlying these effects involves activation of phosphoinositide 3-kinase, mitogen-activated protein kinase, and Src tyrosine kinases. These data indicate that a novel action of leptin in the CNS is to facilitate hippocampal synaptic plasticity via enhanced NMDA receptor-mediated Ca^{2+} influx. Impairment of this process may contribute to the cognitive deficits associated with diabetes mellitus.

Key words: leptin; NMDA; Ca^{2+} imaging; PI 3-kinase; MAPK; Src tyrosine kinase

It is well established that leptin regulates food intake and body weight via activation of the hypothalamic form of the leptin receptor Ob-Rb (Tartaglia et al., 1995). This action may, in part, be attributed to leptin inhibition of glucose-receptive (GR) hypothalamic neurons, via activation of ATP-sensitive potassium (K_{ATP}) channels (Spanswick et al., 1997). However, there is growing evidence that leptin receptor immunoreactivity (Hakansson et al., 1998) and mRNA (Mercer et al., 1996) are also expressed in areas of the CNS not associated with energy balance, such as the hippocampus, suggesting that leptin may have additional functions in these brain regions.

The leptin receptor is a member of the class I cytokine receptor superfamily that signal via janus tyrosine kinases (JAKs). Several pathways are activated by JAKs, including insulin receptor substrate (IRS) proteins, and phosphoinositide 3-kinase (PI 3-kinase) is one of many proteins activated downstream of IRS-1 (Myers and White, 1996). Indeed, leptin signals via PI 3-kinase in insulinoma cells (Harvey et al., 2000), hepatocytes (Zhao et al., 2000), and muscle cells (Berti et al., 1997). Activation of mitogen-activated protein kinase (MAPK) has also been implicated as a signaling intermediate for leptin in various cells (Takahashi et al., 1997; Tanabe et al., 1997). Hippocampal neurons also express high levels of PI 3-kinase (Folli et al., 1994) and MAPK (Fiore et al., 1993).

In the hippocampus, NMDA receptors are necessary for the induction of long-term potentiation (LTP) in most pathways (Bliss and Collingridge, 1993). Src tyrosine kinases can enhance NMDA receptor function (Salter, 1998), and tyrosine kinase inhibitors block the induction of LTP (O'Dell et al., 1991). Thus, stimulation of tyrosine kinase-dependent pathways is one way of modulating hippocampal LTP. Because leptin can activate a number of signaling pathways associated with hippocampal LTP, we hypothesized that leptin could modulate NMDA receptor-dependent hippocampal synaptic plasticity. In this study we show that leptin converts short-term potentiation (STP) into LTP, via facilitation of NMDA receptor-mediated synaptic transmission.

MATERIALS AND METHODS

Materials. Recombinant human leptin (Sigma, St. Louis, MO), prepared in 0.01–0.02% bovine serum albumin as a carrier was used in all experiments. LY 294002, wortmannin, PPI, PP2, PP3, Lavendustin A and B, U0126, and U0124 (all from Calbiochem, La Jolla, CA), tetrodotoxin, 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[F]quinoxaline (NBQX), and PD 98059 (Tocris Cookson, Ballwin, MO), and picrotoxin (Sigma) were obtained commercially.

Ca^{2+} imaging techniques. Hippocampal cultures were prepared using standard procedures as described previously (Rae et al., 2000). A conventional imaging system (Perkin-Elmer, Emeryville, CA) was used to measure changes in $[Ca^{2+}]_i$. Cells were incubated with the Ca^{2+}-sensitive dye fura-2 AM (6 μM; 40–60 min; room temperature). Dye

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loading and subsequent experiments were performed in HEPES-buffered saline (in mM: NaCl 135, HEPES 10, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.0, and D-glucose 25, pH 7.4) supplemented with 0.5 μM TTX, at room temperature (22–25°C). NMDA responses were evoked in neurons perfused with Mg^{2+}-free HEPES-buffered saline supplemented with 10 μM glycine and 0.5 μM TTX. Ratiometric images (350/380 nm) were collected at 2 sec intervals, and data were expressed as changes in fluorescence ratio. Compounds were applied directly to the perfusate. Data were derived from somas of individual hippocampal neurons (6–14 d in culture), identified by their morphological and functional characteristics (Rae et al., 2000). All n values represent data (number of cells) obtained from a minimum of three different cultures prepared from different rats.

Electrophysiology experiments. Rat hippocampal slices (300 μm) were prepared (from 3–5 week old rats) using standard techniques and perfused with a medium (bubbled with 95% O_2 and 5% CO_2) containing (mM): NaCl 124, KCl 3, $NaHCO_3$ 26, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 1, and D-glucose 10. Whole-cell recordings from stratum pyramidale of area CA1 were obtained using electrodes (4–6 MΩ) containing (in mM): Cs^+ methanesulphonate 130, NaCl 5, $CaCl_2$ 1, HEPES 5, EGTA 1, Mg-ATP 5, Na-GTP 0.5, and QX-314 5, pH 7.3. All recordings were made in the presence of 50 μM picrotoxin to block $GABA_A$ receptor-mediated currents. Intracellular cesium ions blocked $GABA_B$ receptor-mediated currents. NMDA receptor-mediated EPSCs were isolated by the addition of NBQX (5 μM). EPSCs were evoked by electrical stimulation at a frequency of 0.2 Hz by a bipolar stimulating electrode placed on Schaffer collateral-commissural fibers, and cells were voltage-clamped at −60 mV. Primed burst stimulation comprised a single shock followed 200 msec later by four consecutive shocks (100 msec apart), at the test intensity (Rose and Dunwiddie, 1986). Synaptic records are the average of two consecutive responses, and stimulus artifacts are blanked for clarity. Recordings were made using an Axopatch 200B amplifier; signals were filtered at 2 kHz, digitized at 10 kHz, and stored on computer. EPSC amplitude and input resistance was monitored throughout experiments. The mean series resistance for all cells was 28 ± 2.4 MΩ (n = 30 cells). Experiments were performed at room temperature (22–25°C).

Electrophysiology of recombinant NMDA receptors expressed in Xenopus *oocytes.* Adult female *Xenopus* laevis were maintained in a temperature- and light-controlled environment. Selected stage V and VI oocytes were injected with cDNAs (20 ng of DNA/cell; NR1/NR2A, 1:1 ratio and NR1/NR2A/Ob-Rb, 1:1:1 ratio). For oocyte expression studies, cDNAs for NR1/NR2A and Ob-Rb were subcloned into cytomegalovirus and pcDNA3 vectors, respectively. Whole-cell currents were recorded from oocytes 1–3 d after injection. Currents were elicited by application of NMDA with glycine (10 μM) at a holding potential of −60 mV. Oocytes were perfused in Mg^{2+}-free, normal frog Ringer's solution consisting of (mM): NaCl 116, KCl 2, $BaCl_2$ 2, and HEPES 5, pH 7.4 with NaOH. For leptin receptor activation, oocytes were incubated in leptin (100 nM) for 10 min.

RESULTS

Leptin converts STP into LTP

In the CA1 region of the hippocampus, the synaptic activation of NMDA receptors and a concomitant rise in postsynaptic $[Ca^{2+}]_i$ are required for the induction of LTP (Bliss and Collingridge, 1983). Under control conditions, NMDA receptors contribute little to basal synaptic transmission, but are activated during high-frequency stimulation (Herron et al., 1986). Exposure of hippocampal CA1 neurons to leptin (50 nM) resulted in a marked facilitation of NMDA receptor-dependent synaptic plasticity. A modest primed-burst stimulation paradigm (Rose and Dunwiddie, 1986) was used to evoke STP, which returned to baseline levels after 30–35 min (n = 5) (Fig. 1*a*). Incubation with leptin (50 nM) immediately before this stimulation paradigm resulted in the conversion of STP into robust LTP (63 ± 1.9% enhancement compared with control at 40 min), lasting up to 60 min (n = 5) (Fig. 1*b*).

Leptin facilitates NMDA-mediated but not AMPA-mediated responses

Because potentiation of NMDA responses can facilitate the induction of LTP (Malenka, 1991), we investigated whether leptin modulates NMDA receptor function. Leptin (50 nM) had a small, inhibitory effect on AMPA receptor-mediated EPSCs, which readily reversed on washout (n = 6) (Fig. 2*a*) and was not accompanied by any change in input resistance (n = 6). In contrast, NMDA receptor-mediated EPSCs were markedly enhanced after exposure to leptin (50 nM) (Fig. 2*b*). This effect was slowly developing (taking 4–6 min to begin), reached a maximum increase of 56 ± 1.7% (10–15 min after the onset of perfusion; n = 5), and was sustained for up to 30 min in the presence of leptin (n = 3). Furthermore, in hippocampal cultures, leptin (5–10 nM) rapidly and reversibly enhanced the increase in $[Ca^{2+}]_i$ induced by NMDA (n = 833) (Fig. 3*a*). At 10 nM, leptin enhanced the NMDA-induced Ca^{2+} rise by 52.3 ± 2.8% (n = 803). This action was reproducible on subsequent second (n = 530) and third (n = 16; data not shown) exposures to leptin, 20–30 min later. In contrast, addition of leptin (10 nM) in the absence of NMDA failed to increase $[Ca^{2+}]_i$ levels (n = 119) (Fig. 3*a*). The ability of leptin to enhance NMDA responses was specific for

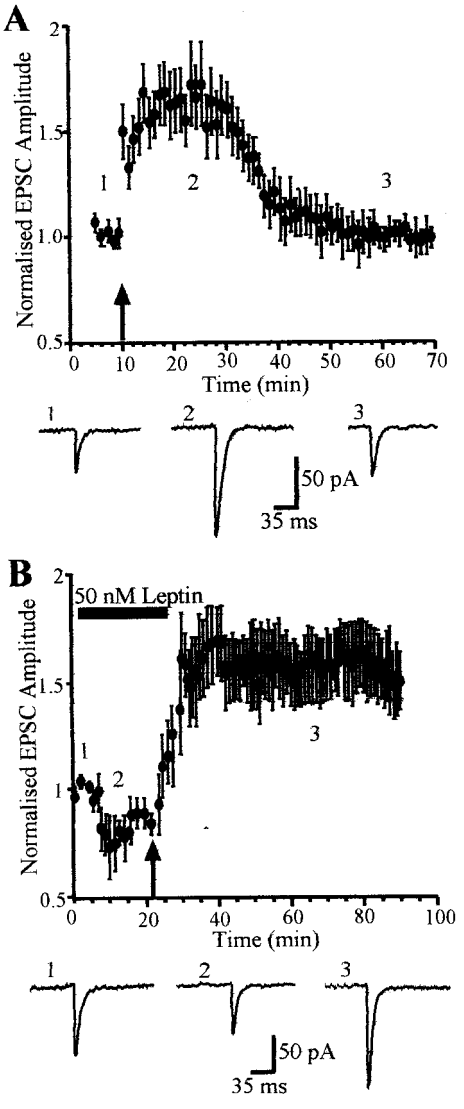

Figure 1. Leptin converts STP into LTP. *A*, Pooled data (n = 5 ± SEM) showing that primed burst stimulation (indicated by the *arrow*) delivered in the absence of leptin induced STP but failed to induce LTP. Synaptic records taken from one experiment show the short-lasting potentiation that returns to baseline at ~30 min later. *B*, During exposure to leptin (50 nM), the same stimulation paradigm resulted in robust LTP (n = 5).

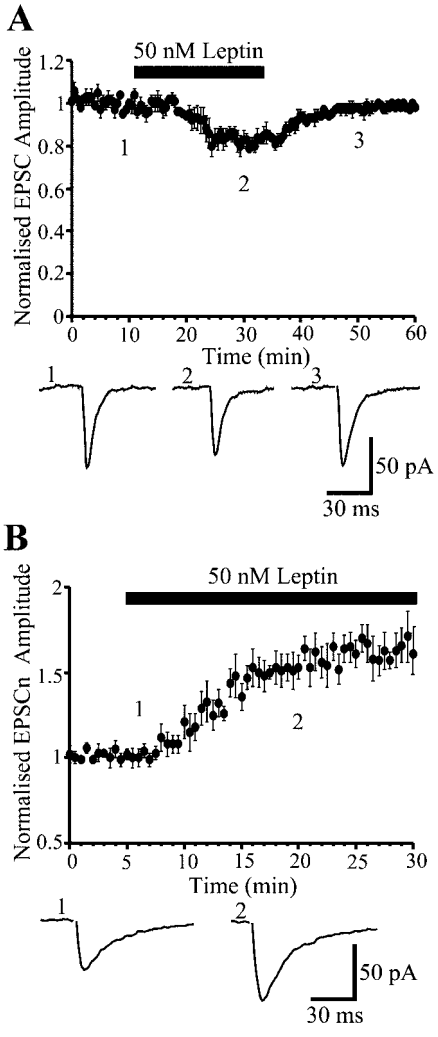

Figure 2. Leptin facilitates NMDA receptor-mediated synaptic transmission. *A*, Pooled data ($n = 6 \pm$ SEM) illustrating that leptin (50 nM) depressed the amplitude of AMPA receptor-mediated EPSCs in a reversible manner. Synaptic records (average of 2 traces) taken from one experiment are illustrated below the plot. *B*, NMDA receptor-mediated synaptic transmission is enhanced by leptin (50 nM). Application of leptin for the time indicated caused a slowly developing facilitation of NMDA EPSCs. The graph illustrates pooled data from five separate experiments.

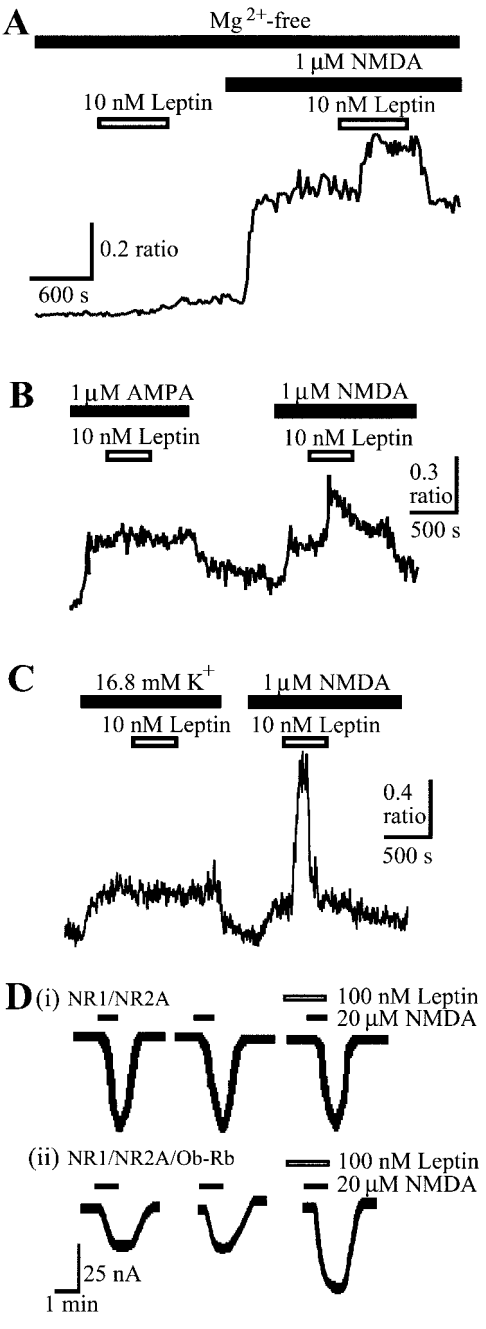

Figure 3. Leptin selectively facilitates NMDA-induced Ca^{2+} rises. *A*, In cells bathed in Mg^{2+}-free medium, addition of leptin (10 nM) alone had no effect on $[Ca^{2+}]_i$ levels. In contrast, in the presence of NMDA (1 μM), which itself increased $[Ca^{2+}]_i$ levels, subsequent addition of leptin (10 nM) caused a further increase in $[Ca^{2+}]_i$. *B*, *C*, Leptin does not enhance AMPA or K^+-induced responses. In the absence of Mg^{2+}, addition of 1 μM AMPA (*B*) or 16.8 mM K^+ (*C*) elevated $[Ca^{2+}]_i$ levels in hippocampal neurons. Subsequent addition of leptin (10 nM) failed to increase $[Ca^{2+}]_i$ further. In the same cells, leptin (10 nM) enhanced the $[Ca^{2+}]_i$ rises induced by NMDA (1 μM). *Di*, In *Xenopus* oocytes expressing NR1/NR2A recombinant channels, leptin (100 nM) failed to enhance NMDA currents (20 μM). However, in oocytes coexpressing NR1/NR2A and Ob-Rb (*Dii*), leptin (100 nM) potentiated currents induced by NMDA (20 μM).

NMDA because comparable rises in $[Ca^{2+}]_i$ induced by AMPA (1 μM; $n = 71$) or high K^+ (10.6 or 16.8 mM; $n = 150$) were not significantly affected by leptin ($p > 0.05$) (Fig. 3*b,c*). To determine whether leptin enhancement of NMDA responses required leptin receptor activation, the actions of leptin were also examined in *Xenopus* oocytes. In oocytes expressing NR1/NR2A alone, application of leptin (100 nM; 10 min) did not enhance the currents evoked by 20 μM NMDA ($n = 3$) (Fig. 3*d*). However, addition of leptin (100 nM; 10 min) to oocytes expressing NR1/NR2A and Ob-Rb facilitated NMDA currents by 50.3 ± 20.5% ($n = 5$) (Fig. 3*d*). These data indicate that leptin, via leptin receptor activation, selectively enhances NMDA receptor function in hippocampal neurons.

Leptin facilitation of NMDA-induced Ca^{2+} rises is prevented by PI 3-kinase and MAPK inhibitors

In insulin-secreting cells (Harvey et al., 2000), hepatocytes (Zhao et al., 2000), and muscle cells (Berti et al., 1997), PI 3-kinase is a key component of leptin receptor-driven signaling pathways. PI 3-kinase also plays a role in synaptic plasticity because the PI 3-kinase inhibitor, wortmannin, blocks hippocampal LTP (Kelly and Lynch, 2000). We therefore examined the effects of specific

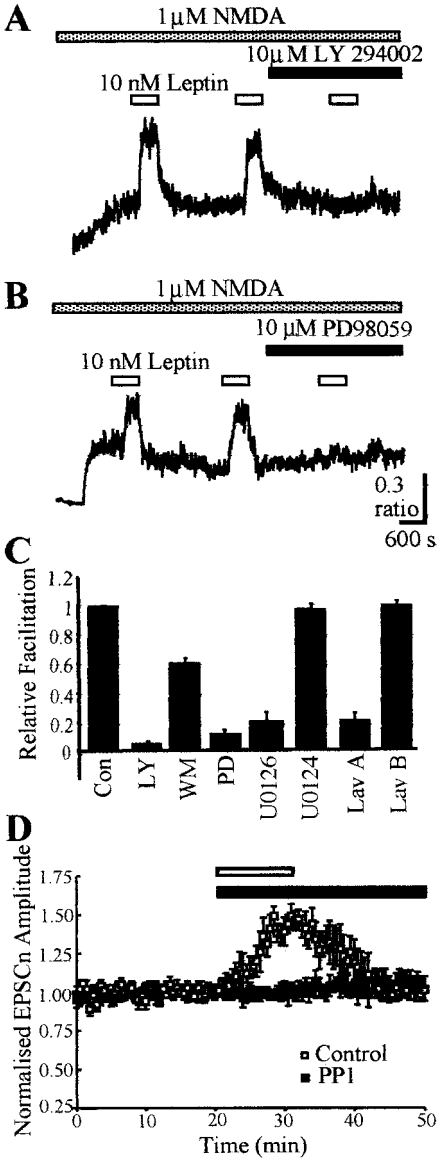

Figure 4. Leptin facilitation of NMDA responses involves activation of PI 3-kinase, Src tyrosine kinase, and MAPK. Leptin facilitation of NMDA responses is attenuated by the PI 3-kinase inhibitor LY 294002 (10 μM; *A*) or the MAPK inhibitor PD98059 (10 μM; *B*). *C*, Pooled data illustrating the degree of facilitation induced by leptin in control conditions ($n = 151$) and in the presence of LY294002 (10 μM; $n = 98$), wortmannin (10 nM; $n = 113$), PD98059 (10 μM; $n = 146$), U0126 (1 μM; $n = 19$), U0124 (1 μM; $n = 15$), lavendustin A (500 nM; $n = 151$), or lavendustin B (500 nM; $n = 15$). *D*, Pooled data ($n = 5 \pm$ SEM) showing that dialysis with PP1 (10 μM; *filled square*) prevented leptin (50 nM; 30 min application; *filled bar*) facilitation of NMDA EPSCs in hippocampal slices. In contrast, after 20 min of dialysis, addition of leptin (50 nM; *open bar*) resulted in reversible enhancement of NMDA EPSCs ($n = 3$).

PI 3-kinase inhibitors, LY 294002 and wortmannin, on responses to leptin. Application of LY 294002 (10 μM) or wortmannin (10 nM) did not effect NMDA responses *per se,* but both agents attenuated the ability of leptin to potentiate NMDA-induced Ca^{2+} rises. Thus, in the presence of LY 294002 (10 μM) or wortmannin (10 nM), the leptin-induced enhancement of NMDA responses was reduced by 97.8 ± 2.1% ($n = 98$; $p < 0.05$) (Fig. 4*a,c*) and 36.8 ± 3.0% ($n = 113$; $p < 0.05$) (Fig. 4*c*), respectively, indicating that a PI 3-kinase-dependent process underlies this action of leptin.

MAPK is also a signaling intermediate for leptin (Takahashi et al., 1997; Tanabe et al., 1997), actions that are sensitive to PD 98059, an inhibitor of MAPK activation. Although PI 3-kinase activates a variety of signaling pathways (Shepherd et al., 1998), it is unclear whether leptin activation of MAPK occurs via a PI 3-kinase-dependent or -independent process. However, leptin stimulation of glucose uptake involves activation of MAPK, as well as PI 3-kinase (Berti and Gammeltoft, 1999), suggesting that MAPK activation requires PI 3-kinase stimulation in some systems. MAPK pathways also play an important role in hippocampal synaptic plasticity (Impey et al., 1999; Rosenblum et al., 2000). Thus, the effects of PD 98059, and U0126, a selective inhibitor of MEK activity, were examined. Incubation with PD98059 (10 μM) had little effect on NMDA responses per se, but it substantially reduced (to 6.3 ± 5.8% enhancement; $n = 146$; $p < 0.01$) the ability of leptin (10 nM) to enhance NMDA responses (Fig. 4*c*). Similarly, U0126 (1 μM) reduced leptin-induced enhancement of NMDA responses by 81 ± 7.8% ($n = 19$) (Fig. 4*c*). In control experiments, previous exposure to U0124 (1 μM; inactive analog of U0126), failed to inhibit leptin facilitation of NMDA-induced Ca^{2+} rises ($n = 15$) (Fig. 4*c*). Thus, blockade of the MAPK pathway also occludes leptin potentiation of NMDA responses in hippocampal neurons.

Src tyrosine kinase inhibitors prevent the effects of leptin

It is well established that Src tyrosine kinases can enhance NMDA receptor function (Yu et al., 1997; Zheng et al., 1998; Salter, 1998). In addition, tyrosine kinase inhibitors block the induction of LTP (O'Dell et al., 1991), and fyn knock-out mice display impaired LTP (Grant et al., 1992), suggesting that tyrosine kinases may be involved in hippocampal LTP. Activation of Src tyrosine kinase, downstream of the focal adhesion kinase CAKβ/Pyk2, has also recently been implicated in this process (Huang et al., 2001). Src tyrosine kinases can also link certain receptors to stimulation of Ras-dependent MAPK (Dikic et al., 1996). Indeed, estrogen enhances NMDA receptor function and LTP in hippocampal neurons via activation of Src tyrosine kinases and MAPK pathways (Bi et al., 2000). Thus, we examined the possible involvement of tyrosine kinases in the actions of leptin. The tyrosine kinase inhibitor lavendustin A (500 nM) had no effect on NMDA responses per se ($n = 151$), but it significantly reduced leptin enhancement of NMDA mediated Ca^{2+} responses (to 12.1 ± 8.7% enhancement; $n = 151$; $p < 0.01$) (Fig. 4*c*). However, the inactive analog lavendustin B (500 nM) did not affect the ability of leptin to facilitate NMDA-induced Ca^{2+} rises ($n = 15$) (Fig. 4*c*). Similarly, in hippocampal slices, whole-cell dialysis with the membrane impermeant Src tyrosine kinase inhibitors PP1 (10 μM; $n = 6$) (Fig. 4*d*) or PP2 (1 μM; $n = 4$; not illustrated), abolished leptin enhancement of NMDA EPSCs. In contrast, dialysis with 1 μM PP3, an inactive analog of PP2, failed to prevent leptin enhancement of NMDA EPSCs ($n = 3$). Thus, after dialysis with PP3 for at least 20 min and after addition of leptin (50 nM; 10 min), NMDA EPSCs were facilitated by 63.2 ± 4.6% ($n = 3$). Furthermore, in parallel experiments, application of leptin (50 nM) after 20 min dialysis resulted in 49.6 ± 4.2% enhancement (10 min after leptin application) of NMDA receptor-mediated EPSCs ($n = 3$) (Fig. 4*d*). These data indicate that leptin facilitation of NMDA receptor-mediated synaptic transmission and NMDA-induced Ca^{2+} rise involves a Src tyrosine kinase-dependent process.

DISCUSSION

Our main finding is that leptin, at concentrations comparable with those circulating in the plasma (Caro et al., 1996) can modulate hippocampal synaptic plasticity, by conversion of STP into LTP. A key process underlying this effect is the enhancement of NMDA responses; a process not only requiring activation of PI 3-kinase, but also MAPK and Src tyrosine kinases. A crucial intracellular process regulating NMDA receptor function is phosphorylation (MacDonald et al., 1989), and both serine–threonine and tyrosine phosphorylation regulate NMDA receptor function. In particular, Src tyrosine kinases can directly phosphorylate NMDA receptor NR2A (Lau and Huganir, 1995) and NR2B (Moon et al., 1994) subunits. Functionally this may be important in hippocampal synaptic plasticity because it has been hypothesized that during LTP induction, Src is rapidly activated leading to enhanced NMDA receptor function (Salter, 1998). However, the mechanisms causing activation of Src during LTP are unknown. One possibility is that leptin released during high-frequency stimulation could facilitate NMDA responses via a Src-dependent pathway. Although leptin mRNA has been detected in the CNS (Morash et al., 1999), it is not clear whether leptin itself is released synaptically. Another possibility is that hormonal leptin released from adipocytes could cross the blood–brain barrier (Banks et al., 1996) and act to modulate the threshold for the induction of synaptic plasticity.

In insulin-secreting cells (Harvey et al., 2000), hepatocytes (Zhao et al., 2000), and muscle cells (Berti et al., 1997), PI 3-kinase is associated with leptin receptor signaling. The present study also indicates that activation of PI 3-kinase underlies the ability of leptin to potentiate NMDA responses. In parallel with other studies (Takahashi et al., 1997; Tanabe et al., 1997), our data also indicates that stimulation of MAPK plays an important role in leptin action. However, further biochemical studies are required to determine whether activation of MAPK and Src by leptin occurs downstream of PI 3-kinase (Bondeva et al., 1998).

There is growing evidence that diabetes mellitus is associated with dementia and cognitive deficits (Gispen and Biessels, 2000). Moreover, diabetic animal models commonly display impairments in spatial learning, with concomitant changes in hippocampal plasticity (Biessels et al., 1996; Kamal et al., 1999), actions thought to be associated with either insulin deficiency or insulin resistance. Obesity, another common feature of diabetes, is attributed to leptin resistance even under conditions of hyperleptinaemia (Sinha et al., 1996). In view of the present findings, leptin resistance may be a crucial factor associated with the cognitive deficits observed in diabetics. In the absence of defects in leptin production or the leptin receptor per se (Considine et al., 1996; Maffei et al., 1996), leptin resistance is likely to reflect compromised intracellular signaling cascades, which would limit the potential for synaptic plasticity and could, in part, underlie the cognitive deficits associated with diabetes mellitus.

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