

# Leptin inhibits epileptiform-like activity in rat hippocampal neurones via PI 3-kinase-driven activation of BK channels

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The obese gene product, leptin is an important circulating satiety factor that regulates energy balance via its actions in the hypothalamus. However, leptin receptors are also expressed in brain regions not directly associated with energy homeostasis, such as the hippocampus. Here, leptin inhibits hippocampal neurones via activation of large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  (BK) channels, a process that may be important in regulating neuronal excitability. We now show that leptin receptor labelling is expressed on somata, dendrites and axons, and is also concentrated at synapses in hippocampal cultures. In functional studies, leptin potently and reversibly reduces epileptiform-like activity evoked in lean, but not leptin-resistant Zucker *fa/fa* rats. Furthermore, leptin also depresses enhanced  $\text{Ca}^{2+}$  levels evoked following  $\text{Mg}^{2+}$  removal in hippocampal cultures. The ability of leptin to modulate this activity requires activation of BK, but not  $\text{K}_{\text{ATP}}$ , channels as the effects of leptin were mimicked by the BK channel activator NS-1619, and inhibited by the BK channel inhibitors, iberiotoxin and charybdotoxin. The signalling mechanisms underlying this process involve stimulation of phosphoinositide 3-kinase (PI 3-kinase), but not mitogen-activated protein kinase (MAPK), as two structurally unrelated inhibitors of PI 3-kinase, LY294002 and wortmannin, blocked the actions of leptin. These data indicate that leptin, via PI 3-kinase-driven activation of BK channels, elicits a novel mechanism for controlling neuronal excitability. As uncontrolled excitability in the hippocampus is one underlying cause of temporal lobe epilepsy, this novel action of leptin could provide an alternative therapeutic target in the management of epilepsy.

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The obese gene product leptin is an important circulating, satiety factor that regulates energy balance via activation of the hypothalamic form of the leptin receptor (Ob-Rb; Jacob *et al.* 1997); an action that has been attributed to inhibition of hypothalamic neurones via ATP-sensitive  $\text{K}^+$  ( $\text{K}_{\text{ATP}}$ ) channel activation (Spanswick *et al.* 1997). However, leptin receptor immunoreactivity (Hakansson *et al.* 1998) and mRNA (Mercer *et al.* 1996) are also expressed in areas of the CNS not directly associated with energy homeostasis, suggesting that leptin has additional functions in these brain regions. Leptin itself crosses the blood–brain barrier and may be released locally in the CNS (Morash *et al.* 1999).

The leptin receptor is a member of the class I cytokine receptor superfamily (Tartaglia *et al.* 1995) that signals via association with janus tyrosine kinases (JAKs). Several pathways are activated by JAKs including insulin receptor substrate (IRS) proteins (Myers & White, 1996), and phosphoinositide 3-kinase (PI 3-kinase) is one protein activated downstream of IRS-1 (Shepherd *et al.* 1998). Indeed, leptin signals via PI 3-kinase in insulinoma cells (Harvey *et al.* 2000 *b*), muscle cells (Berti *et al.* 1997) and hepatocytes (Zhao *et al.* 2000). The main function of PI

3-kinase is to convert phosphatidylinositol bisphosphate ( $\text{PtdIns}(4,5)\text{P}_2$ ) into phosphatidylinositol trisphosphate ( $\text{PtdIns}(3,4,5)\text{P}_3$ ; Shepherd *et al.* 1998). Signalling cascades activated downstream of PI 3-kinase that utilise  $\text{PtdIns}(3,4,5)\text{P}_3$  as a second messenger include mitogen-activated protein kinase (MAPK), stress-activated protein kinase 2 (SAPK2) and protein kinase B. Indeed, activation of MAPK has also been implicated as a signalling intermediate for leptin (Takahashi *et al.* 1997; Tanabe *et al.* 1997). Hippocampal neurones also express high levels of IRS-1, PI 3-kinase (Folli *et al.* 1994) and MAPK (Fiore *et al.* 1993). Indeed, leptin modulates NMDA receptor function in the hippocampus via a PI 3-kinase- and MAPK-dependent process (Shanley *et al.* 2001).

We have shown recently that leptin inhibits hippocampal neurones via activation of large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  (BK) channels (Shanley *et al.* 2002). Neuronal BK channel activity is highly dependent on the levels of intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) at any given voltage (Latorre, 1989). BK channels are activated during an action potential when the membrane potential depolarises and  $[\text{Ca}^{2+}]_i$  rises, and are critical in determining action potential firing rates as well as burst firing patterns. As

leptin activates BK channels in hippocampal neurones (Shanley *et al.* 2002), we hypothesised that leptin, via BK channel stimulation, could modulate aberrant synaptic activity in hippocampal neurones. In this study we show, using hippocampal slices and cultured neurones, that leptin inhibits epileptiform-like activity via PI 3-kinase-driven BK channel activation. This process represents a novel mechanism for controlling hippocampal excitability. Some of these data have been published previously in abstract form (Shanley *et al.* 2000).

## METHODS

### Materials

Recombinant human leptin (Sigma, St Louis, MO, USA) prepared in 0.01–0.02 % bovine serum albumin as a carrier was used in all experiments. LY 294002, wortmannin, (Calbiochem, La Jolla, CA, USA); tetrodotoxin, PD 98059 (Tocris Cookson, Baldwin, MO, USA); NS-1619 (Biomol); nifedipine, D-APV, diazoxide, glipizide (Sigma); and iberiotoxin and charybdotoxin (Alomone Labs, Israel) were all obtained commercially.

### Cell culture

Cultures of hippocampal neurones were prepared using standard procedures as described previously (Irving & Collingridge, 1998), but were maintained in serum replacement medium (SR2, Sigma). In brief, rat pups 1–3 days old were killed by cervical dislocation and hippocampi removed. The hippocampi were washed in standard Hepes-buffered saline (HBS) comprising (mM): NaCl 135; KCl 5; CaCl<sub>2</sub> 1; MgCl<sub>2</sub> 1; Hepes 10; D-glucose 25; at pH 7.4. The hippocampi were then treated with a mixture of protease type XIV and type X (both at 0.5 mg ml<sup>-1</sup>; Sigma) for 25 min at room temperature. Dissociated cells were plated onto sterile culture dishes, pretreated with poly-L-lysine (20 µg ml<sup>-1</sup> for 1–2 h). Cultures were maintained in a humidified atmosphere of 5 % CO<sub>2</sub> at 37 °C for up to 2 weeks.

### Immunocytochemistry

A goat polyclonal antibody directed against the C-terminal domain of the leptin receptor (Santa Cruz Biotechnology; Hakansson *et al.* 1998) was used. All immunocytochemical procedures were carried out in HBS. Prior to labelling, hippocampal cultures were fixed with 4 % paraformaldehyde and permeabilised with 0.1 % triton X-100. Cells were then exposed to 10 % blocking milk for 15 min. Hippocampal cultures were incubated with the leptin receptor antibody overnight at 4 °C. Immunostaining was visualised by the addition of an Alexa 488-conjugated donkey anti-goat secondary antibody (Molecular Probes). In dual labelling experiments, monoclonal markers for MAP2, GAP43 and synapsin I (all from Sigma) were visualised with a Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Immuno-Research). In the absence of primary antibody, no labelling was observed following incubation with any of the secondary antibodies. In control experiments, leptin receptor immunoreactivity was blocked by prior incubation of primary antibody with control peptide (200 µg ml<sup>-1</sup>). A laser scanning confocal imaging system (Bio-Rad Microradiance or Zeiss LSM 510) was used for image acquisition. Laser lines of 488 and 543 nm were used to excite Alexa 488 and Cy3, respectively. With the Bio-Rad system, sequential images were obtained (Kalman average of 4–8 individual scans) and the two parent images were merged using Lasershar software. With the Zeiss confocal microscope, dual-labelling images were obtained in multi-tracking mode using a 15 s scan speed.

### Ca<sup>2+</sup> imaging techniques

A conventional digital epifluorescence imaging system (12 bit; Perkin-Elmer, Emeryville, CA, USA) mounted on an Olympus BX50WI microscope (× 40 objective) was used to measure changes in [Ca<sup>2+</sup>]<sub>i</sub>. Cells were incubated with the Ca<sup>2+</sup>-sensitive dye fura-2 AM (6 µM; 40–60 min; room temperature). Dye loading and subsequent experiments were performed in Hepes-buffered saline (mM): NaCl 135, Hepes 10, KCl 5, CaCl<sub>2</sub> 1, glycine 0.01 and D-glucose 25 at pH 7.4, with or without added MgCl<sub>2</sub> (1 mM), at room temperature (22–25 °C). Ratiometric images (350/380 nm excitation; 510 nm emission) were collected at 2 s intervals and data are expressed as changes in fluorescence ratio.

Compounds were applied directly to the perfusate. Data were derived from the somata of individual hippocampal neurones (6–14 days in culture), identified by their morphological and functional characteristics (Rae *et al.* 2000). Exposure of cultures to Mg<sup>2+</sup>-free Hepes buffered saline resulted in an increase in intracellular Ca<sup>2+</sup> levels associated with the appearance of spontaneous Ca<sup>2+</sup> oscillations. The effects of leptin and all the other agents on this activity were quantified by measuring the mean fluorescence ratio over a 2–3 min period immediately prior to leptin and/or agent addition and for a similar time period in the presence of leptin and/or agent. All *n* values represent data (number of cells) obtained from a minimum of three different cultures prepared from different rats.

### Electrophysiological recordings

Rat hippocampal slices (400 µm) were prepared from 3–4-week-old Zucker rats using standard techniques and perfused with artificial cerebrospinal fluid (aCSF), bubbled with 95 % O<sub>2</sub> and 5 % CO<sub>2</sub> and containing (mM): NaCl 124, KCl 3, NaHCO<sub>3</sub> 26, NaHPO<sub>4</sub> 1.25, CaCl<sub>2</sub> 2, MgSO<sub>4</sub> 1 and D-glucose 10. Epileptogenic conditions were obtained by maintaining hippocampal slices in aCSF with no added Mg<sup>2+</sup>. Extracellular recording pipettes (5–15 MΩ) filled with aCSF were positioned in stratum pyramidale of CA1 and stimuli were delivered every 2 min via a stimulating electrode placed in the stratum radiatum. Stimulation intensity was set to two times the intensity to yield maximum population spikes (range, 40–90 V for 0.2 ms duration). Such stimulation resulted in the generation of spontaneous 'interictal' epileptiform activity, following perfusion of Mg<sup>2+</sup>-free media for 2–3 h (Kohling *et al.* 2000). Epileptiform events were continuously monitored on a chart recorder and the frequency of interictal activity was analysed off line. Experiments were performed at room temperature (22–25 °C) and the CA3 region was left intact to increase the levels of spontaneous activity. Extracellular field recordings were made using an Axopatch 200B amplifier; signals were filtered at 2 kHz and stored on a computer.

### Analysis

All data are expressed as means ± S.E.M. and statistical analyses were performed using Student's unpaired *t* test, for comparison of means or ANOVA (analysis of variance) for comparisons between multiple groups. *P* < 0.05 was considered significant.

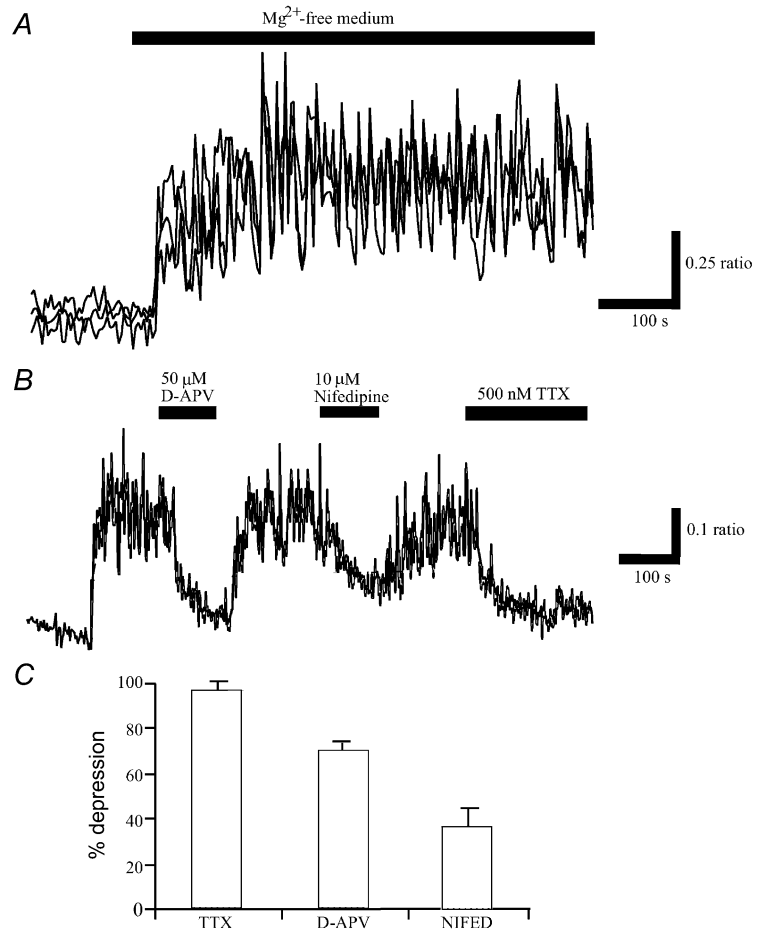
## RESULTS

### Characterisation of the spontaneous Ca<sup>2+</sup> transients induced by Mg<sup>2+</sup>-free conditions

Epileptiform events are associated with elevations in intracellular Ca<sup>2+</sup> levels (Badea *et al.* 2001). In cultured hippocampal neurones, low or zero Mg<sup>2+</sup> conditions result in phasic elevations in [Ca<sup>2+</sup>]<sub>i</sub>; associated with the induction

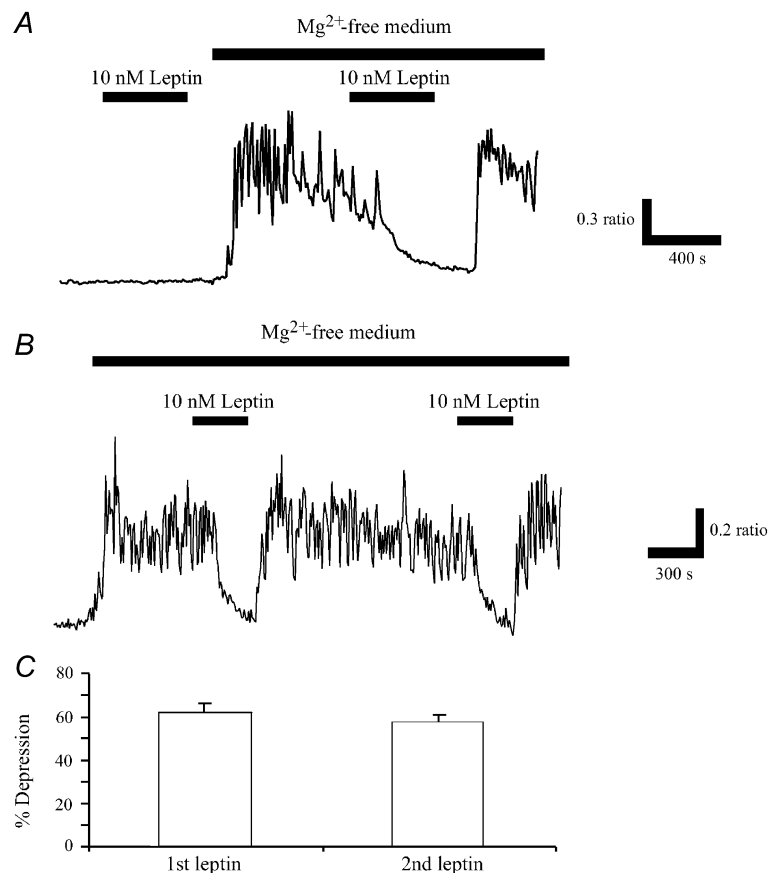
**Figure 1. Exposure of hippocampal cultures to Mg<sup>2+</sup>-free conditions induces spontaneous Ca<sup>2+</sup> oscillations**

A, perfusion of hippocampal cultures with Mg<sup>2+</sup>-free HEPES-buffered saline resulted in a rise in Ca<sup>2+</sup> levels that was accompanied by the appearance of synchronised oscillations in Ca<sup>2+</sup>. Traces are derived from the somata of individual neurones within a field of cells. B, following generation of the spontaneous Ca<sup>2+</sup> transients, application of TTX (500 nM) for the time indicated resulted in complete inhibition of the enhanced Ca<sup>2+</sup> load associated with Mg<sup>2+</sup> removal. Application of the NMDA receptor antagonist, D-APV (50 μM) or VGCC blocker, nifedipine (10 μM), attenuated the spontaneous activity in a reversible manner. C, pooled data illustrating the relative depressions of mean Ca<sup>2+</sup> load in Mg<sup>2+</sup>-free medium following the addition of TTX (1 μM; n = 84), D-APV (50 μM; n = 80) or nifedipine (10 μM; n = 28). Thus, these synaptically driven Ca<sup>2+</sup> transients required glutamatergic synaptic transmission as well as the influx of Ca<sup>2+</sup> via VGCCs.



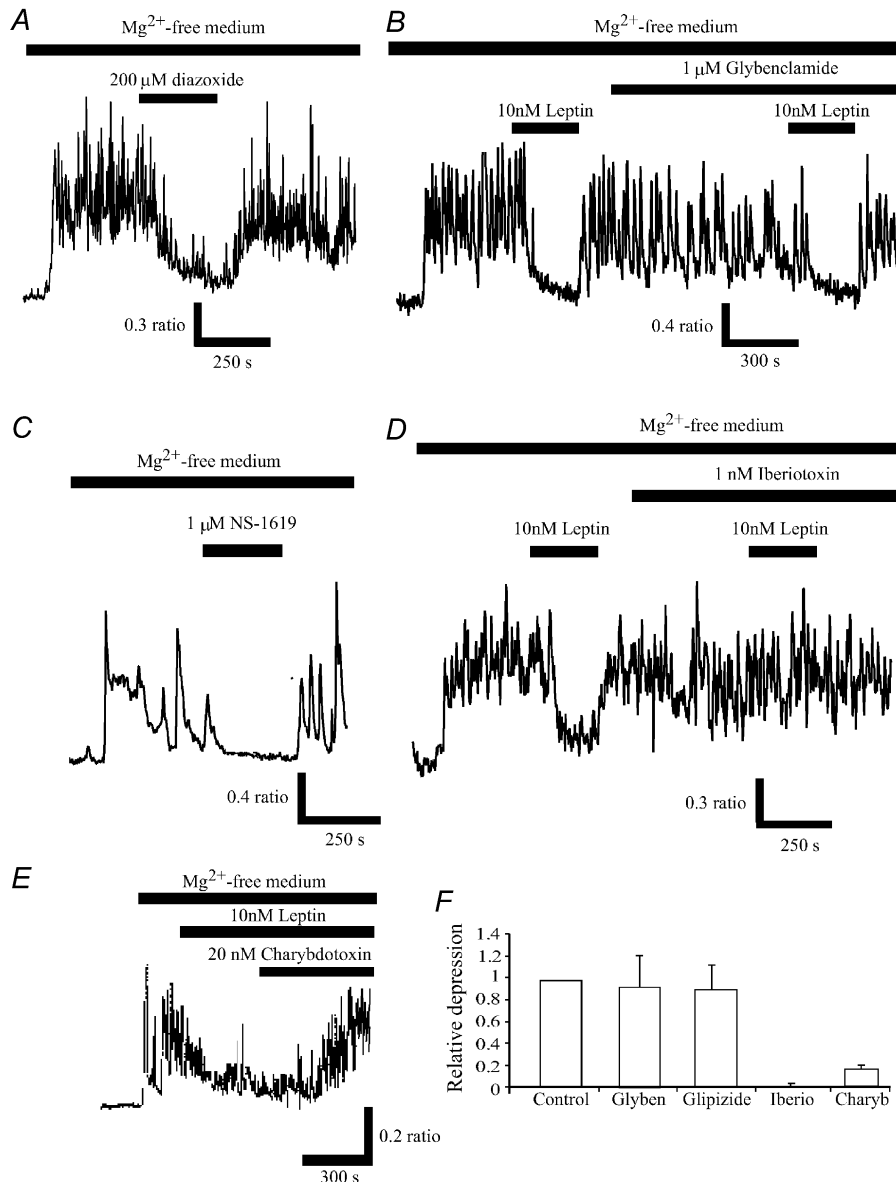
**Figure 2. Leptin inhibits the enhanced Ca<sup>2+</sup> levels evoked following Mg<sup>2+</sup> removal**

A, in control conditions (Mg<sup>2+</sup>-containing solution), application of leptin (10 nM) for the time indicated by the bar, had no effect the basal levels of Ca<sup>2+</sup>. However, following perfusion of Mg<sup>2+</sup>-free medium, which itself resulted in the generation of spontaneous Ca<sup>2+</sup> oscillations, addition of leptin (10 nM) caused a rapid inhibition of the Ca<sup>2+</sup> levels. This action of leptin was readily reversed on washout. B, the ability of leptin to inhibit this enhanced Ca<sup>2+</sup> load was readily reproducible as subsequent application of leptin (10 nM), 20–30 min after the first exposure, resulted in a comparable response. C, pooled data illustrating that the relative depressions of mean Ca<sup>2+</sup> load in Mg<sup>2+</sup>-free medium induced by sequential applications of leptin do not differ significantly.



of recurrent bursts of synaptic activity (Abele *et al.* 1990; McLeod *et al.* 1998). This process, which requires NMDA receptor activation, action potential propagation and the influx of  $\text{Ca}^{2+}$  via voltage-gated  $\text{Ca}^{2+}$  channels, resembles seizure-like activity observed in brain slices following  $\text{Mg}^{2+}$  removal (Abele *et al.* 1990; McLeod *et al.* 1998; Delorenzo *et al.* 1998). Perfusion of hippocampal cultures with glycine-containing,  $\text{Mg}^{2+}$ -free medium resulted in an

increase in  $[\text{Ca}^{2+}]_i$  that was subsequently followed by the generation of spontaneous  $\text{Ca}^{2+}$  oscillations (Fig. 1A). In order to determine if the properties of these oscillations were consistent with those described previously (Abele *et al.* 1990; Shen *et al.* 1996; McLeod *et al.* 1998), the effects of inhibitors of NMDA receptors,  $\text{Na}^+$  channels and voltage-gated  $\text{Ca}^{2+}$  channels (VGCC) were examined. Application of the selective  $\text{Na}^+$  channel blocker, tetrodotoxin (TTX);



**Figure 3. Inhibition of  $\text{Ca}^{2+}$  levels by leptin involves BK, but not K<sub>ATP</sub>, channel activation**

A, following perfusion of  $\text{Mg}^{2+}$ -free medium and the generation of the spontaneous  $\text{Ca}^{2+}$  oscillations, addition of the K<sub>ATP</sub> channel opener, diazoxide (200  $\mu\text{M}$ ) reversibly depressed the mean enhancement of  $\text{Ca}^{2+}$  levels following  $\text{Mg}^{2+}$  removal. C, similarly, the BK channel activator, NS-1619 also depressed  $\text{Ca}^{2+}$  levels in a readily reversible manner. B and D, the K<sub>ATP</sub> channel inhibitor, glybenclamide did not affect the leptin-modulation of  $\text{Ca}^{2+}$  levels, whereas the selective BK channel inhibitor, iberiotoxin completely blocked the actions of leptin. E, leptin-induced depression of the spontaneous  $\text{Ca}^{2+}$  transients was readily reversed by the addition of charybdotoxin (20 nM). F, pooled data illustrating the relative depressions of mean  $\text{Ca}^{2+}$  load in  $\text{Mg}^{2+}$ -free medium by leptin in control conditions, and in the presence of glybenclamide (1  $\mu\text{M}$ ), glipizide (1  $\mu\text{M}$ ), iberiotoxin (1 nM) or charybdotoxin (20 nM).

500 nM) abolished the mean, global enhancement in  $\text{Ca}^{2+}$  levels associated with the oscillations ( $n = 84$ ; Fig. 1B and C). Furthermore, addition of the competitive NMDA receptor antagonist, D(-)-2-amino-5-phosphonopentanoic acid (D-AP5, 50  $\mu\text{M}$ ) or the L-type VGCC blocker, nifedipine (10  $\mu\text{M}$ ) reduced the  $\text{Ca}^{2+}$  levels by  $70.5 \pm 3.9\%$  ( $n = 80$ ) and  $36.7 \pm 9.3\%$  ( $n = 28$ ), respectively (Fig. 1B and C). Thus, these data indicate that the characteristics of the spontaneous  $\text{Ca}^{2+}$  transients in the present study parallel those reported previously as they are dependent on NMDA receptor activation, influx of  $\text{Ca}^{2+}$  via VGCCs and action potential propagation.

### Leptin inhibits $\text{Ca}^{2+}$ levels in $\text{Mg}^{2+}$ -free medium via activation of BK, but not $\text{K}_{\text{ATP}}$ channels

Application of leptin (0.1–30 nM; 5 min) rapidly (within 1–2 min) reduced the enhancement in global  $\text{Ca}^{2+}$  levels following 10–20 min exposure to  $\text{Mg}^{2+}$ -free medium by  $62.6 \pm 2.4\%$  ( $n = 416$ ; Fig. 2A), in a readily reversible manner. This action of leptin was reproducible as subsequent re-application of leptin, 15–20 min later induced a comparable response ( $n = 225$ ; Fig. 2B and C). This protocol was therefore used to investigate the cellular mechanisms underlying leptin action.

Previous studies have shown that potassium channel activators, such as diazoxide, can modulate aberrant synaptic activity, evoked following  $\text{Mg}^{2+}$  removal (Abele & Miller, 1990). Furthermore, leptin activates  $\text{K}_{\text{ATP}}$  channels in insulin-secreting cells (Harvey *et al.* 1997; Keiffer *et al.* 1997) and GR hypothalamic neurones (Spanswick *et al.* 1997). Thus, in the next series of experiments the role of  $\text{K}_{\text{ATP}}$  channels in leptin action was examined. In agreement with previous studies (Abele & Miller, 1990), the  $\text{K}_{\text{ATP}}$  channel opener, diazoxide mimicked the actions of leptin, such that addition of diazoxide (200  $\mu\text{M}$ ) inhibited the enhanced  $\text{Ca}^{2+}$  levels by  $53.7 \pm 2.8\%$  ( $n = 57$ ; Fig. 3A). This action of diazoxide was evident within 1–2 min of application and reversed on washout. Application of the selective  $\text{K}_{\text{ATP}}$  channel inhibitor, glybenclamide (1–10  $\mu\text{M}$ ) had no effect on the mean  $\text{Ca}^{2+}$  levels *per se*, such that the mean ratio values obtained in the absence and presence of glybenclamide were  $1.07 \pm 0.05$  and  $1.07 \pm 0.04$ , respectively ( $n = 92$ ;  $P > 0.05$ ; Fig. 3B). Furthermore, exposure of the cultures to glybenclamide (1  $\mu\text{M}$ ) for at least 20 min prior to the addition of leptin (10 nM), had little or no effect on the ability of leptin to inhibit the enhanced  $\text{Ca}^{2+}$  levels associated with exposure to  $\text{Mg}^{2+}$ -free medium (Fig. 3B and F). Thus, in the presence of glybenclamide, leptin (10 nM) lowered the enhanced  $\text{Ca}^{2+}$  levels by  $38 \pm 3.3\%$  compared with  $44 \pm 3.5\%$  in its absence ( $n = 57$ ;  $P > 0.05$ ). Similarly, addition of another  $\text{K}_{\text{ATP}}$  channel inhibitor, glipizide (1  $\mu\text{M}$ ) had no effect on the ability of leptin to modulate the mean  $\text{Ca}^{2+}$  load in  $\text{Mg}^{2+}$ -free medium (Fig. 3F). Thus, leptin lowered  $\text{Ca}^{2+}$  levels by  $39.0 \pm 1.7$  and  $37.2 \pm 2.8\%$  in the absence and

presence of 1  $\mu\text{M}$  glipizide, respectively ( $n = 51$ ;  $P > 0.05$ ), indicating that a process independent of  $\text{K}_{\text{ATP}}$  channel activation underlies leptin action.

We have shown previously that leptin inhibits hippocampal neurones via activation of BK channels (Shanley *et al.* 2002). In the next series of experiments we examined whether BK channel activation is also involved in the actions of leptin in hippocampal cultures. Application of the BK channel activator, NS-1619 (1  $\mu\text{M}$ ; 5 min; Olesen *et al.* 1994) mimicked the actions of leptin, causing  $50 \pm 6.4\%$  inhibition of the mean  $\text{Ca}^{2+}$  load in  $\text{Mg}^{2+}$ -free medium ( $n = 31$ ; Fig. 3C). Application of the BK channel inhibitor, iberiotoxin (1 nM; Galvez *et al.* 1990) itself had no effect on  $\text{Ca}^{2+}$  levels *per se*, with mean ratio values of  $0.92 \pm 0.07$  and  $0.90 \pm 0.08$ , in the absence and presence of iberiotoxin, respectively ( $n = 42$ ;  $P > 0.05$ ). However, the ability of leptin to modulate intracellular  $\text{Ca}^{2+}$  levels was completely blocked by prior incubation with this agent ( $n = 42$ ; Fig. 3D and F). Thus, the mean depressions induced by leptin in the absence and presence of 1 nM iberiotoxin were  $45 \pm 2.3$  and  $2.4 \pm 1.8\%$ , respectively ( $n = 42$ ;  $P < 0.05$ ). Similarly, incubation with charybdotoxin (20 nM) significantly reduced the leptin-induced inhibition of  $\text{Ca}^{2+}$  levels (Fig. 3F), such that the mean depressions induced by leptin (10 nM) were  $47.7 \pm 3.6$  and  $9.9 \pm 6.5\%$ , in the absence and presence of charybdotoxin, respectively ( $n = 48$ ;  $P < 0.05$ ). Furthermore, application of charybdotoxin (20–50 nM) 10–12 min after the addition of leptin, rapidly reversed the leptin-induced depression of the enhanced  $\text{Ca}^{2+}$  levels in  $\text{Mg}^{2+}$ -free medium ( $n = 65$ ; Fig. 3E), such that the mean depression induced by leptin was reduced from  $69.7 \pm 5.3\%$  (10 min after exposure to leptin) to  $26.2 \pm 7.3\%$ , 13–15 min after exposure to 20 nM charybdotoxin ( $n = 65$ ;  $P < 0.05$ ). Together these data indicate that leptin inhibition of spontaneous, synaptically driven  $\text{Ca}^{2+}$  transients in cultured hippocampal neurones involves activation of BK, but not  $\text{K}_{\text{ATP}}$  channels.

### Leptin inhibits $\text{Ca}^{2+}$ levels via a PI 3-kinase, but not by a MAPK, driven process

There is growing evidence that PI 3-kinase is a key component of leptin receptor signalling pathways in insulinoma cells (Harvey *et al.* 2000b), cardiac cells (Berti *et al.* 1997) and hepatocytes (Zhao *et al.* 2000). A PI 3-kinase-dependent process also underlies leptin-induced activation of  $\text{K}_{\text{ATP}}$  channels in hypothalamic neurones (Mirshamsi & Ashford, 2001) and leptin stimulation of BK channels in CA1 hippocampal neurones (Shanley *et al.* 2002). Thus, we next determined whether activation of this enzyme plays a role in leptin action. Application of the PI 3-kinase inhibitors, LY 294002 (10  $\mu\text{M}$ ;  $n = 108$ ; Vlahos *et al.* 1994) or wortmannin (10–50 nM;  $n = 77$ ; Powis *et al.* 1994) for 25 min had no effects on the mean  $\text{Ca}^{2+}$  levels in  $\text{Mg}^{2+}$ -free medium (Fig. 4A and B). Thus, the mean ratio

values obtained in control conditions were  $0.57 \pm 0.05$  (LY294002) and  $0.98 \pm 0.09$  (wortmannin), whereas following incubation with the PI 3-kinase inhibitors for at least 15 min, the ratio values were  $0.56 \pm 0.06$  ( $n = 108$ ; LY294002;  $P > 0.05$ ) and  $0.93 \pm 0.08$  ( $n = 77$ ; wortmannin;  $P > 0.05$ ). However, both agents substantially reduced the effects of leptin (Fig. 4C). Thus, the mean depressions induced by leptin (10 nM) in the presence of LY 294002 (10  $\mu$ M) or wortmannin (10 nM) were  $3.3 \pm 2.2\%$  ( $n = 38$ ;  $P < 0.05$ ) and  $5.5 \pm 1.7\%$  ( $n = 67$ ;  $P < 0.05$ ), respectively (Figure 4C).

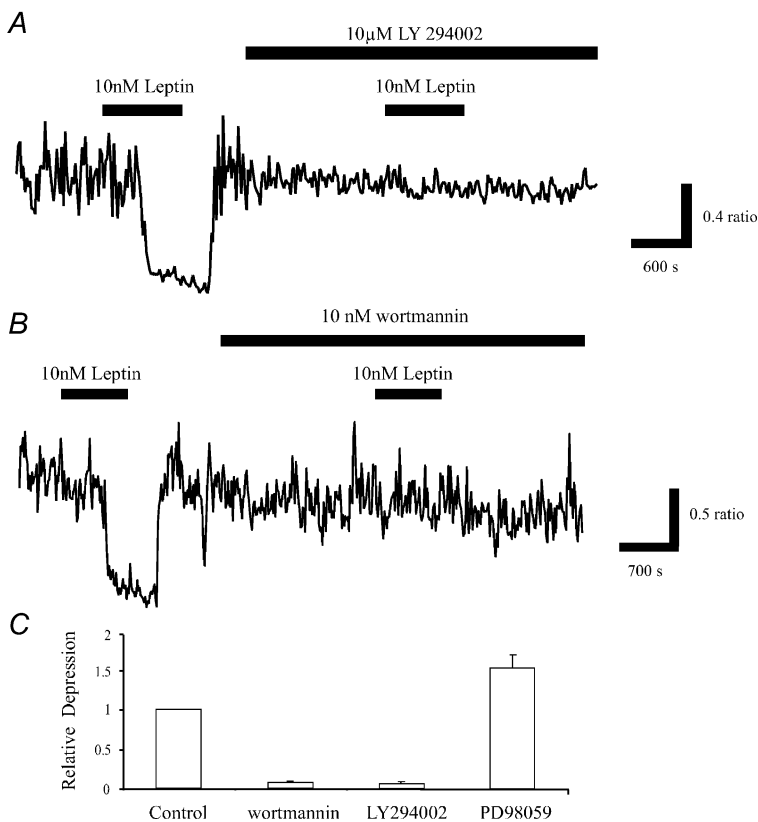
Activation of MAPK has also been implicated as a signalling intermediate for leptin in various cell types (Takahashi *et al.* 1997; Tanabe *et al.* 1997), actions sensitive to PD98059, an inhibitor of MAPK activation (Alessi *et al.* 1995). Leptin facilitation of NMDA receptor-mediated responses also requires activation of MAPK (Shanley *et al.* 2001). Thus, the effects of PD 98059 on the ability of leptin to lower  $Ca^{2+}$  levels were examined. Application of PD 98059 (10  $\mu$ M) for 15–20 min caused little or no effect on the  $Ca^{2+}$  levels in  $Mg^{2+}$ -free medium, with mean ratio values of  $0.70 \pm 0.08$  and  $0.69 \pm 0.09$  obtained in the absence and presence of PD 98059, respectively ( $n = 100$ ;  $P > 0.05$ ). However, subsequent addition of leptin (10 nM) in the continued presence of PD98059 resulted in  $62.2 \pm 5.3\%$  inhibition of the mean  $Ca^{2+}$  load compared with  $47.8 \pm 5.1\%$  inhibition in control conditions ( $n = 60$ ;  $P < 0.01$ ; Fig. 4C). Thus these data indicate not only that activation of MAPK pathways

are not essential for leptin action, but also that the ability of leptin to modulate the mean  $Ca^{2+}$  load is enhanced following blockade of MAPK activation.

### Leptin reduces epileptiform-like activity in hippocampal slices

As BK channels are particularly suited for controlling neuronal excitability, and epileptiform burst-like activity can be modulated by BK channel activity (Alger & Williamson, 1988; Golding *et al.* 1999; Shao *et al.* 1999), we next determined if leptin was also able to modulate epileptiform-like activity evoked in hippocampal slices. Furthermore, in order to address if the actions of leptin are due to activation of the leptin receptor, we compared the effects of leptin in hippocampal slices from control lean and obese Zucker *fa/fa* rats. In this rodent model of obesity, the *fa* mutation gives rise to a leptin-resistant state (Ahima & Flier, 2000). In these experiments, slices were bathed in  $Mg^{2+}$ -free solution for at least 3 h prior to recording. In slices from Zucker lean rats, the frequency of interictal activity was significantly lower than in age-matched Zucker obese *fa/fa* rats. Thus, the mean frequency of interictal events in lean and *fa/fa* rats was  $0.037 \pm 0.003$  Hz ( $n = 4$ ) and  $0.101 \pm 0.031$  Hz ( $n = 4$ ), respectively ( $P < 0.05$ ), suggesting that animals with leptin receptor signalling deficiency show increased neuronal excitability.

In slices from lean animals, application of leptin (50 nM) inhibited the mean frequency of interictal events by 67% from  $0.037 \pm 0.003$  to  $0.012 \pm 0.003$  Hz (8 min after



### Figure 4. Leptin inhibition of $Ca^{2+}$ levels requires stimulation of PI 3-kinase

A, application of leptin (10 nM) for the time indicated caused reversible inhibition of the enhanced  $Ca^{2+}$  load in  $Mg^{2+}$ -free medium. Exposure of the neurones to the PI 3-kinase inhibitor, LY 294002 (10  $\mu$ M) for at least 15 min had no effect on the enhanced  $Ca^{2+}$  levels. However, subsequent addition of leptin in the continued presence of LY 294002 failed to inhibit  $Ca^{2+}$  levels. B, similarly, wortmannin (10 nM), another PI 3-kinase inhibitor had no effect on the enhanced  $Ca^{2+}$  levels, but it did significantly reduce the effects of leptin. C, pooled data illustrating the effects of leptin on the mean  $Ca^{2+}$  load in  $Mg^{2+}$ -free medium in control conditions, and in the presence of wortmannin (10 nM;  $n = 67$ ), LY 294002 (10  $\mu$ M;  $n = 38$ ) or the inhibitor of MAPK activation, PD 98059 (10  $\mu$ M;  $n = 51$ ).

addition of leptin;  $n = 4$ ;  $P < 0.05$ : Fig. 5A and B). This action of leptin readily reversed on washout, with recovery to  $0.039 \pm 0.004$  Hz (10–15 min later;  $n = 4$ ). In order to address the locus of leptin action, the effects of leptin on evoked field excitatory postsynaptic potentials (fEPSPs) were monitored simultaneously. In 4 slices, leptin induced a small but significant depression of the slope and amplitude of evoked fEPSPs ( $n = 4$ ; Fig. 5E and F). Thus, application of leptin (50 nM) caused  $17.8 \pm 3.1$  and  $18.8 \pm 2.8$  % depression in the fEPSP slope and amplitude, respectively ( $n = 4$ ;  $P < 0.05$ ). Leptin also lowered the level of excitability by reducing the number of population spikes evoked by single shock stimulation (Fig. 5E).

In order to address whether leptin receptor activation was required for leptin action, the effects of leptin were also investigated in slices obtained from Zucker *fa/fa* rats. Addition of leptin (50–100 nM; for up to 20 min) had no effect on interictal activity evoked in slices from age-matched obese Zucker *fa/fa* rats ( $n = 4$ ; Fig. 5C and D), such that the mean frequency of interictal events was  $0.10 \pm 0.04$  Hz in control conditions and  $0.11 \pm 0.06$  Hz following 15 min incubation with leptin ( $n = 4$ ;  $P > 0.05$ ).

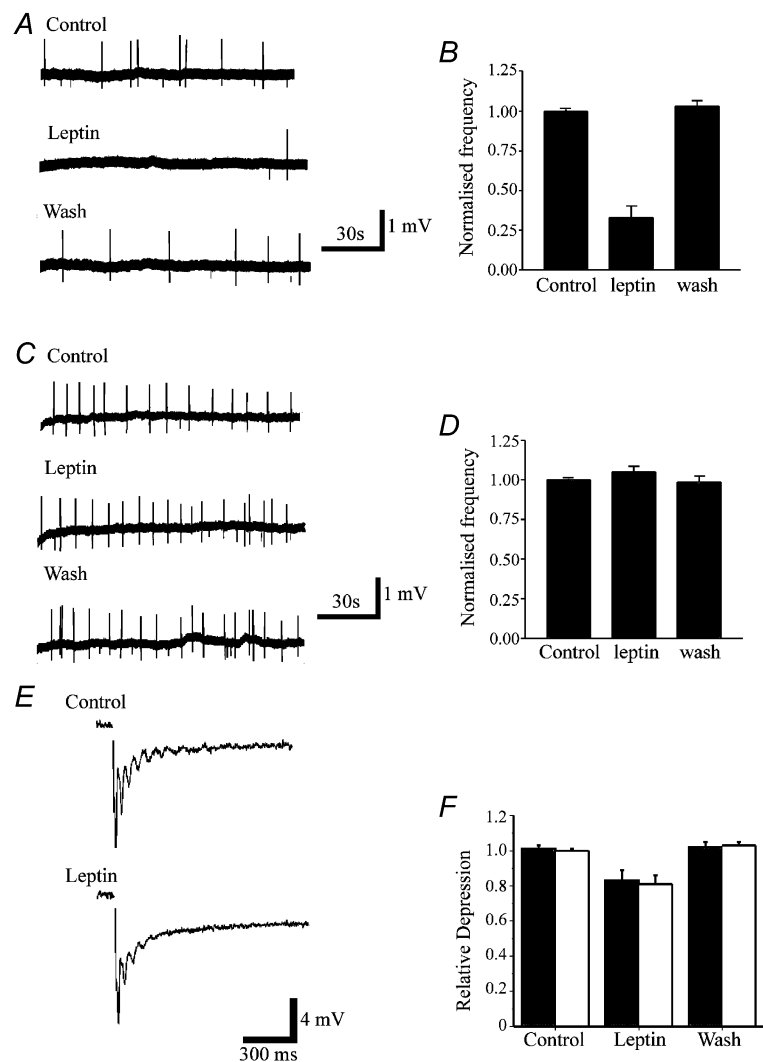
Together these data indicate not only that animals with leptin resistance display enhanced neuronal excitability, but also that leptin, via leptin receptor activation, is a potent modulator of epileptiform-like activity.

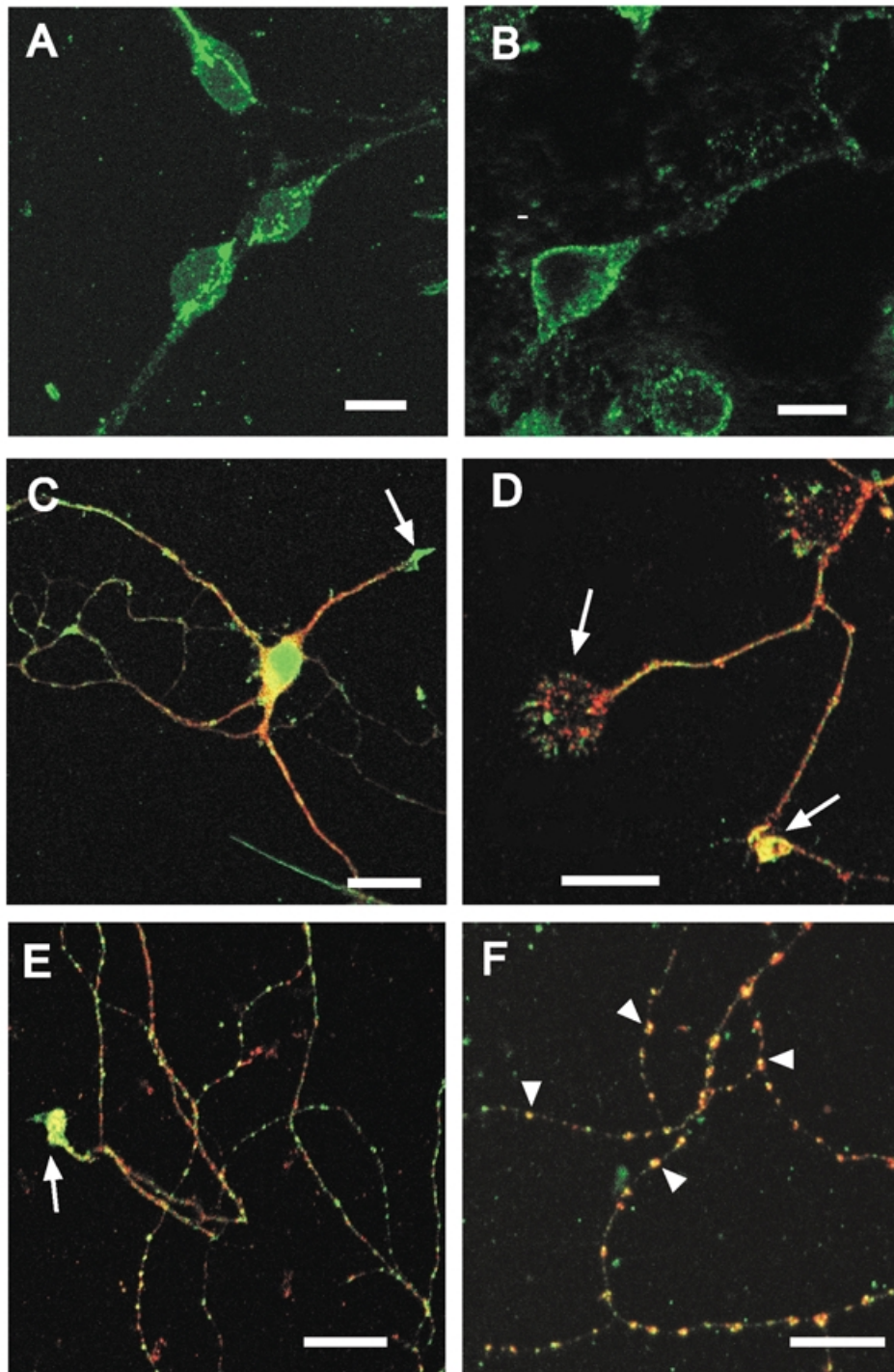
### Functional localisation of leptin receptors in cultured hippocampal neurones

Previous studies have shown that leptin receptor immunoreactivity (Hakansson *et al.* 1998) and mRNA (Mercer *et al.* 1996) are widely expressed throughout the hippocampal formation. In this study, the functional localisation of leptin receptors was assessed in cultured hippocampal neurones using dual labelling immunocytochemical techniques. Leptin receptor labelling was present on cultured hippocampal neurones (Fig. 6). In young cultures (6–8 days) labelling was primarily associated with the somato-dendritic region and putative growth cones (axonal and dendritic). However, in older cultures punctate labelling was also detected along fine processes (Fig. 6). At somata, leptin receptor immunoreactivity was associated with the plasma membrane. In some somata bright, punctate leptin receptor labelling was also observed within the cytoplasm (Fig. 6), which is likely to relate to intra-

#### Figure 5. Leptin inhibits interictal activity in Zucker lean, but not obese *fa/fa* rats

A, sample traces of extracellularly recorded spontaneous interictal events evoked in a hippocampal slice obtained from a Zucker lean rat. Leptin (50 nM) reduced the frequency of interictal activity in a readily reversible manner. B, plot of the pooled data illustrating the normalised interictal frequency in control conditions, in the presence of leptin (50 nM) and following washout ( $n = 4$ ). C, sample traces of extracellularly recorded hippocampal epileptiform interictal activity in slices from obese Zucker *fa/fa* rats. Addition of leptin (50 nM) failed to affect the frequency of interictal events. D, pooled data illustrating the normalised frequency of interictal events in slices from *fa/fa* rats, in control conditions, in the presence of leptin and following washout ( $n = 4$ ). E, sample records of evoked fEPSPs obtained in control conditions and following exposure to 50 nM leptin. Note that leptin not only reduced the slope and peak amplitude of the fEPSP, but it also reduced the number of population spikes. F, pooled data illustrating the relative depressions of the slope (■) and amplitude (□) of fEPSPs evoked in control conditions, following exposure to leptin (50 nM) and on washout.





**Figure 6. Leptin receptor localisation on cultured hippocampal neurones**

*A* and *B*, leptin receptor immunoreactivity on neuronal somata, illustrating punctate labelling within the cytoplasm (*A*) and labelling associated with the plasma membrane (*B*). *A* is a Z-projection of a series of confocal images taken at 1  $\mu\text{m}$  intervals, *B* is a single confocal section. Primary antibody labelling was visualised with an Alexa 488-conjugated donkey anti-goat secondary antibody (green). *C*, *D*, *E* and *F*, merged, single plane confocal images comparing leptin receptor labelling with monoclonal markers (red; Cy3) for soma/dendrites (MAP2; *C*), axons (GAP43, *D*) or synaptic terminals (synapsin 1; *E* and *F*). The yellow areas correspond to points of overlap. In these images, the confocal gain and/or laser intensity was increased to facilitate the visualisation of fine processes. Leptin receptor immunoreactivity was associated with synaptic markers in older cultures (9–14 days, *E* and *F*). In the zoomed image (*F*), arrowheads show examples of co-localisation with synapsin 1 at individual puncta. Strong leptin receptor immunoreactivity was also associated with dendritic (*C*) and axonal (*D* and *E*) growth cones (arrows). Scale bars are 10  $\mu\text{m}$  (*A*, *B*, *D*, *E* and *F*) and 20  $\mu\text{m}$  (*C*).



cellular sites of leptin receptor synthesis and metabolism. Comparison of leptin receptor immunoreactivity with monoclonal antibody markers for axons (GAP-43) and soma/dendrites (MAP2) demonstrated leptin receptor expression within these different neuronal compartments (Fig. 6). Dual labelling with an antibody against synapsin 1 indicated that the clusters of leptin receptor immunolabelling on fine processes were associated with synaptic terminals (Fig. 6). This is likely to reflect expression at presynaptic terminals as leptin receptor labelling in fine processes is associated with GAP-43, and follows the path of fibres expressing synapsin 1 (Irving *et al.* 2000).

## DISCUSSION

It is well established that the obese gene product leptin plays a major role in regulating energy balance via its actions in the hypothalamus (Elmqvist *et al.* 1999). This process may be attributable, at least in part, to inhibition of GR hypothalamic neurones via stimulation of  $K_{ATP}$  channels (Spanswick *et al.* 1997). However, there is growing evidence that this hormone can have other functions. Indeed, leptin is thought to play an essential role in both immune (Fantuzzi & Faggioni, 2000) and reproductive function (Caprio *et al.* 2001), is an important factor controlling bone formation (Takeda & Karsenty 2001) and may modulate synaptic plasticity (Shanley *et al.* 2001). In this study, we provide further evidence of a role for leptin in the CNS that is unrelated to hypothalamic control of energy balance. Using immunocytochemical approaches, we demonstrate that leptin receptor immunoreactivity is present on hippocampal neurones. The pattern of labelling is consistent with the expression of functional receptors on soma and proximal dendrites and also at presynaptic terminals. We show that leptin inhibits epileptiform-like synaptic activity in hippocampal neurones, via BK channel activation, indicating that activation of this leptin receptor-driven signalling is a novel pathway for modulating hippocampal excitability.

Previous studies have indicated that leptin receptor immunoreactivity (Hakansson *et al.* 1998) as well as leptin receptor mRNA (Mercer *et al.* 1996), are expressed in hippocampal slices. The present study indicates that leptin receptor labelling is localised throughout cultured hippocampal neurones, with immunoreactivity present on somata, dendrites and axons, and is also concentrated at points of synaptic contact and at growth cones. Thus, leptin receptors are well positioned to regulate neuronal excitability in hippocampal neurones. Our data indicate that leptin reduced the frequency of interictal activity induced in the CA1 region of hippocampal slices, in a readily reversible manner. Furthermore, hippocampal slices from Zucker *fa/fa* rats exposed to  $Mg^{2+}$ -free conditions displayed an increased frequency of interictal activity relative to lean controls, suggesting that animals

with leptin receptor signalling deficiencies have an increased degree of neuronal excitability.

Leptin also lowered the enhanced  $Ca^{2+}$  levels evoked following  $Mg^{2+}$  removal in hippocampal cultures, but had no effect on the basal levels of  $Ca^{2+}$ . The ability of leptin to modulate this activity in hippocampal neurones parallels its reported actions on spontaneous  $Ca^{2+}$  oscillatory activity evoked in hypothalamic neurones (Glaum *et al.* 1996). Consistent with previous studies (Abele & Miller, 1990), application of the  $K_{ATP}$  channel opener, diazoxide inhibited the mean  $Ca^{2+}$  load in a readily reversible manner. Similarly, addition of the BK channel activator, NS-1619 inhibited this activity. However, the modulatory actions of leptin were prevented by the BK channel inhibitors, iberiotoxin and charybdotoxin, but not by the  $K_{ATP}$  channel blocker glybenclamide or glipizide, indicating that leptin receptor-driven activation of BK, but not  $K_{ATP}$ , channels underlies this process.

In hippocampal pyramidal neurones, postsynaptic BK channels are activated during an action potential by membrane depolarisation, together with a rise in  $[Ca^{2+}]_i$ . The resulting potassium current is largely responsible for repolarisation of action potentials and the generation of the fast after-hyperpolarisation (Storm, 1987; Lancaster & Nicoll, 1987), which in turn delays recovery of the membrane potential to firing threshold. Thus, postsynaptic BK channel activity is vital in regulating the level of neuronal excitability, which determines action potential firing rate and burst firing patterns. Indeed, a role for BK channels in regulating epileptiform-like activity has been suggested previously (Alger & Williams, 1988; Golding *et al.* 1999; Shao *et al.* 1999). Recent evidence also indicates that presynaptic BK channels may influence synaptic transmission at CA1 synapses, but only under conditions of enhanced excitability, for example induced by the  $K^+$  channel blocker, 4-AP (Hu *et al.* 2001). Thus, the role of BK channels in hippocampal function is greatly influenced by their subcellular location.

In the present study, leptin receptor immunoreactivity was detected both at presynaptic terminals and somatodendritic regions. Previous studies have shown that BK channels have a similar localisation (Hu *et al.* 2001), suggesting that leptin could theoretically influence hippocampal hyperexcitability, via BK channel stimulation, at either location. In hippocampal slices, leptin markedly inhibited epileptiform-like activity, but only exerted a small depressant effect on evoked field potentials (in  $Mg^{2+}$ -free medium). Our previous studies have indicated that at the cellular level, leptin hyperpolarises CA1 pyramidal neurones via stimulation of postsynaptic BK channels (Shanley *et al.* 2002), thus moving the membrane potential of these neurones away from their firing threshold. We have also reported recently that leptin induces a small reduction in the amplitude of evoked EPSCs (Shanley *et al.*

2001), an action that is independent of any change in input resistance. Thus, it is likely that both pre- and post-synaptic actions of leptin on CA1 pyramidal neurones act in concert to inhibit unregulated excitability in the hippocampus. However, given the proportionately greater effect of leptin on spontaneous interictal activity in comparison with evoked synaptic responses, the postsynaptic actions of leptin are likely to be the more important mechanism underlying the effects of leptin on epileptiform-like events. The mechanisms underlying leptin modulation of EPSCs is unclear at this stage. Studies using blockers of BK channels suggest that they do not regulate transmitter release under basal (low frequency) conditions (Hu *et al.* 2001). However, the present work suggests that the effects of leptin involve activation of BK channels, whose actions may be manifest at the presynaptic terminal under more physiological conditions.

A noteworthy observation in the present study concerns the lack of effect of BK channel blockers on the enhanced  $\text{Ca}^{2+}$  levels in  $\text{Mg}^{2+}$ -free medium. This activity has been shown to reflect synchronous burst firing within populations of cultured neurones leading to somatic depolarisation and an influx of intracellular  $\text{Ca}^{2+}$  (Shen *et al.* 1996). Given that BK channels modulate bursting, it might be expected that BK channel inhibitors would enhance this activity. The lack of effect of iberiotoxin and charybdotoxin on the aberrant synaptic activity may reflect the rapidly desensitising nature of BK channels, such that their activity declines during repetitive firing (Shao *et al.* 1999). Thus, the sustained bursting induced in  $\text{Mg}^{2+}$ -free medium may no longer be influenced by 'conventional' action potential-dependent activation of BK channels. However, leptin, which activates BK channels via a  $\text{Ca}^{2+}$ -independent mechanism (Shanley *et al.* 2002), is still effective.

There is growing evidence that PI 3-kinase is a key component of leptin receptor signalling in peripheral cells including insulin-secreting cells (Harvey *et al.* 2000b), myotubes (Berti *et al.* 1997) and hepatocytes (Zhao *et al.* 2000). Recent evidence also indicates that PI 3-kinase activation is required for leptin-induced facilitation of NMDA receptor function in hippocampal neurones (Shanley *et al.* 2001) and activation of hypothalamic  $\text{K}_{\text{ATP}}$  channels by leptin (Mirshamsi & Ashford, 2001). Furthermore, leptin stimulation of BK channels in hippocampal neurones involves activation of a PI 3-kinase-driven process (Shanley *et al.* 2002). Similarly, in the present study the ability of leptin to modulate the  $\text{Ca}^{2+}$  activity in hippocampal cultures required stimulation of PI 3-kinase. Activation of MAPK by leptin has also been reported in a variety of peripheral cell types (Takahashi *et al.* 1997; Tanabe *et al.* 1997) and leptin-induced enhancement of NMDA responses in hippocampal neurones involves MAPK activation (Shanley *et al.* 2001).

In contrast, in the present study the effects of leptin were not attenuated by PD98059, indicating that leptin inhibition of the raised  $\text{Ca}^{2+}$  levels in  $\text{Mg}^{2+}$ -free medium involves activation of a MAPK-independent pathway. The present data does however indicate that the ability of leptin to modulate this enhanced  $\text{Ca}^{2+}$  load was significantly enhanced in the presence of PD98059, suggesting that leptin receptor-driven MAPK activation possibly acts as a negative feedback mechanism to regulate the actions of leptin.

One function of PI 3-kinase is to promote the conversion of  $\text{PtdIns}(4,5)\text{P}_2$  into  $\text{PtdIns}(3,4,5)\text{P}_3$  (Shepherd *et al.* 1998). There is also growing evidence that inositol phospholipids closely associate with the actin cytoskeleton, where they can modulate a variety of proteins (see Janmey, 1998 for review). Altering the integrity of cytoskeletal networks can also modulate the activity of a variety of ion channels. Indeed, leptin activation of  $\text{K}_{\text{ATP}}$  channels in insulinoma cells involves PI 3-kinase-driven disruption of the actin cytoskeleton (Harvey *et al.* 2000a). Activation of other cytokine or growth factor receptors can also lead to cytoskeletal organisation (Goh *et al.* 1997), via PI 3-kinase-dependent mechanisms (Kim & Feldman, 1998). Thus, it is possible that the ability of leptin to activate BK channels in hippocampal neurones involves a change in the dynamics of the cytoskeleton. In support of this possibility, the activity of human BK channels expressed in meningioma cells is influenced by changes in cytoskeletal architecture (Kraft *et al.* 2000). However, further experiments are required to explore whether this mechanism underlies leptin modulation of hippocampal BK channels.

In conclusion, we have shown that the obese gene product leptin, via PI 3-kinase driven activation of BK channels, inhibits different forms of epileptiform-like activity evoked in hippocampal neurones. It is well established that reducing the levels of extracellular  $\text{Mg}^{2+}$  elicits epileptic discharges in brain slice preparations (Kohr & Heinemann, 1989) and intense synaptic activity in neuronal cultures (Robinson *et al.* 1993). The aberrant synaptic activity that develops is thought to limit the viability of the neurones and can ultimately lead to paroxysmal neuronal firing and cell death (Abele *et al.* 1990; Rose *et al.* 1990). Thus, the ability of leptin to potently inhibit the mean  $\text{Ca}^{2+}$  load associated with this aberrant synaptic activity suggests that leptin is a novel neuroprotective agent and that the leptin receptor-driven signalling pathway may be a useful target in the treatment of epilepsy and certain neurodegenerative disorders.

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