Research

The beneficial effects of leptin on REM sleep deprivation-induced cognitive deficits in mice

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Leptin, a 167 amino acid peptide, is synthesized predominantly in the adipose tissues and plays a key role in the regulation of food intake and body weight. Recent studies indicate that leptin receptor is expressed with high levels in many brain regions that may regulate synaptic plasticity. Here we show that deprivation of rapid eye movement (REM) sleep resulted in impairment of both cue and contextual fear memory. In parallel, surface expression of GluR1 was reduced in the amygdala. Intraperitoneal injection of leptin to the REMD mice rescued memory impairment and reversed surface GluR1 reduction. Using whole-cell recording to evaluate the synaptic function of the thalamus–lateral amygdala (LA) pathway, we found a decrease in frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) concomitant with reduced AMPA/NMDA ratios in the REMD mice. By contrast, paired-pulse facilitation (PPF) was increased. The effects of REMD on mEPSCs and AMPA/NMDA ratio could be reversed by leptin treatment, whereas on PPF it could not. Phosphatase and tensin homolog (PTEN), a dual protein/lipid phosphatase, down-regulates the effect of the PI-3 kinase pathway. Fear conditioning increased whereas REMD led to a decrease in the phosphorylated states of PTEN, Akt, and glycogen synthase kinase-3 β (GSK3 β), and the effects of REMD were reversed by leptin. These results suggest that both pre- and post-synaptic functions of the thalamus–LA pathway were altered by fear conditioning and REMD in opposite directions. Leptin treatment reversed REMD-induced memory deficits primarily by a postsynaptic action by restoring surface expression of GluR1 without affecting PPF.

[Supplemental material is available for this article.]

There is ample evidence suggesting that sleep is necessary for the processes of memory consolidation and a good night's sleep improves retention of long-term memory in both humans and rodents (Maquet 2001; Stickgold et al. 2001; Walker 2008; Poe et al. 2010; McCoy and Strecker 2011). This idea implies that patterns of neuronal activity present in waking are reactivated, analyzed, and integrated into memory networks during subsequent REM sleep (Graves et al. 2001; Benington and Frank 2003). Consistent with this idea, increase in REM sleep has been observed following successful task acquisition (Lucero 1970; Smith and Rose 1996). In addition, REM sleep deprivation at certain post-training times impairs memory for recently acquired tasks (Fishbein 1971; Smith and Rose 1996; Graves et al. 2003; Havekes et al. 2012). Furthermore, long-term potentiation (LTP) of excitatory synaptic transmission in the hippocampus, a candidate mechanism for learning and memory (Bliss and Collingridge 1993), is reduced by sleep deprivation (Campbell et al. 2002; Davis et al. 2003; Havekes et al. 2012).

Pavlovian fear conditioning occurs during the convergence of pathways transmitting the conditioned stimulus (CS) and unconditioned stimulus (US) to the LA. Excitatory pyramidal neurons in the amygdala use glutamate as neurotransmitter (McDonald and Augustine 1993; Sah et al. 2003). Associative activation of CS and US pathways which accompany behavioral learned fear resulted in LTP of synaptic transmission from auditory

thalamus and cortex to the LA (McKernan and Shinnick-Gallagher 1997; Rogan et al. 1997) and increased the synaptic GluR1 subunit of AMPA receptors (AMPA) (Rumpel et al. 2005; Yeh et al. 2006). LTP is occluded in amygdalar slices from fear conditioned animals (Tsvetkov et al. 2002; Schroeder and Shinnick-Gallagher 2005), suggesting that synaptic plasticity occurs in the LA during fear learning. Furthermore, interference with synaptic incorporation of GluR1 receptors in the LA impairs LTP and fear memory formation (Rumpel et al. 2005; Sigurdsson et al. 2007). Thus, AMPAR insertion into synaptic membrane underlies amygdalar synaptic plasticity and fear memory formation.

Leptin, a 167 amino acid peptide, is synthesized predominantly in the adipose tissues and plays a key role in the regulation of food intake and body weight (Spiegelman and Flier 2001). Leptin receptors, however, are expressed with high levels in many extra-hypothalamic brain regions, including the hippocampus, brain stem, cortex, amygdala, and cerebellum (Håkansson et al. 1998), implying that leptin may modulate other brain functions as well as regulate energy homeostasis (Banks 2004). Indeed, the magnitude of LTP was increased and the performance of memory tasks was improved following administration of leptin into the hippocampus (Farr et al. 2004; Wayner et al. 2004). Exposure of hippocampal neurons to leptin resulted in a rapid increase in the density and motility of dendritic filopodia as well as the number of excitatory synapses (O'Malley et al. 2007). Furthermore, leptin rapidly increased GluR1 surface expression in adult hippocampus (Moult et al. 2010).

We have previously shown that memory for newly acquired information was impaired following a specific period of REMD that was associated with an increase in a dual protein/lipid phos-

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phatase PTEN. Importantly, intra-amygdalar administration of antisense but not sense or scrambled oligonucleotides for PTEN prevented REMD-induced impairment of fear memory, suggesting that REMD interfered with the process of memory retention via activation of PTEN (Su et al. 2004). In the present study, we have demonstrated that leptin rescues REMD-induced impairment of fear memory consolidation. We further investigated the mechanisms underlying the action of leptin.

Results

REM sleep deprivation impairs consolidation of fear memory

Mice were given five pairings of tone and footshock. Immediately thereafter, they were divided into sleep deprivation control (SDC), REM sleep deprivation (REMD), and experimental control groups. The mice in the SDC group were restricted in a large platform where they could fall asleep. REM sleep was prevented in a 24-h period 60 min after the training session by putting the mice in a small platform (REMD group). The mice in the experimental control group remained in the home cage without being restricted in a sleep deprivation platform. As shown in Figure 1A, SDC and REMD groups were able to acquire tone–shock association (freezing responses: Control, $74.2 \pm 5.6\%$, $n = 23$; SDC, $78.3 \pm 4.8\%$, $n = 23$; REMD, $68.7 \pm 4.8\%$, $n = 23$, $F_{(2,66)} = 0.904$, $P > 0.1$).

Figure 1. REM sleep deprivation impairs consolidation of fear memory. (A) Mice were given five pairings of tone (CS) and footshock and were able to acquire tone–shock association. (B) Twenty-four hours after conditioning, the mice were tested for memory retention in a new chamber with CS. The freezing response in the REMD group was significantly lower compared to those of control and SDC groups. (***) $P < 0.001$ vs. SDC group. (C) Twenty-four hours after conditioning, the mice were tested for memory retention in the conditioning chamber without CS. The freezing response in the REMD group was significantly lower compared to those of control and SDC groups. (***) $P < 0.001$ vs. SDC group.

Figure 2. Leptin rescued REMD-induced impairment of fear memory. Mice received five tone–footshock pairings and were injected intraperitoneally with saline, 1 mg, 2 mg, or 4 mg/kg leptin before REMD. Cue (A) and contextual (B) freezing responses were measured 1 h after REMD. The 2 mg/kg and 4 mg/kg leptin groups differed from the saline group and less freezing occurred in the low-dose group than in the high-dose group, indicating a dose-dependent effect. (**) $P < 0.01$, (***) $P < 0.001$ vs. saline.

However, when tested 24 h later, mice in the REMD group ($19.6 \pm 4.6\%$, $n = 23$) displayed a significantly lower cue freezing response as compared to SDC ($61.1 \pm 4.7\%$, $n = 23$) and control ($77.5 \pm 2.5\%$, $n = 23$) groups ($F_{(2,66)} = 53.38$, $P < 0.001$) (Fig. 1B). Similarly, the REMD group ($9.2 \pm 2.0\%$, $n = 23$) also exhibited lower contextual freezing response as compared to SDC ($50.7 \pm 5.1\%$, $n = 23$) and control ($69.0 \pm 2.8\%$, $n = 23$) groups ($F_{(2,66)} = 73.86$, $P < 0.001$) (Fig. 1C). These results suggest that REMD impaired both cue and contextual fear memory formation.

Leptin restores REMD-induced memory deficits

We determined whether leptin affected REMD-induced impairment of fear memory. Mice received five tone–footshock pairings and were injected intraperitoneally with saline, 1 mg, 2 mg, or 4 mg/kg leptin before REMD. The doses of leptin used in the present study have been shown to decrease infarct volume and neurologic defects and to improve regional cerebral blood flow after brain injury (Zhang et al. 2007). One-way ANOVA showed that there were significant differences among the four groups in freezing responses ($F_{(3,43)} = 8.465$, $P < 0.001$), and Newman–Keuls post hoc tests revealed that 2 mg/kg and 4 mg/kg leptin groups differed from the saline group ($P < 0.01$ and $P < 0.001$, respectively). Furthermore, less freezing occurred in the low-dose group than in the high-dose group, indicating a dose-dependent effect (Fig. 2A). It was noted that there was no difference in freezing response between SDC and 4 mg/kg leptin mice ($P > 0.1$), suggesting that leptin was able to completely restore REMD-induced impairment of cue fear memory. Similarly, less contextual freezing occurred in the low-dose leptin group than in the high-dose leptin group ($F_{(3,43)} = 8.492$, $P < 0.001$) (Fig. 2B) and there was no difference in freezing response between SDC and 4 mg/kg leptin mice ($P > 0.1$).

We examined the effect of leptin on open-field behaviors. One-way ANOVA showed that there were no significant differences among saline and three leptin treatments in the distance moved

Figure 3. Leptin restored REMD-induced decrease in surface expression of GluR1. (A) Representative blots and mean $\pm$ SEM of surface (left column) and total (right column) GluR1 immunoreactivities from naïve, unpaired, SDC, and REMD mice. BLA tissues were dissected out and surface receptors were labeled with biotin 1 h after REMD. (***) $P < 0.001$ vs. naïve, (###) $P < 0.001$ vs. SDC. (B) Conditioned mice were assigned to SDC, REMD, or leptin (4 mg/kg) groups. Cell extracts of the BLA were prepared and surface (left column) and total (right column) GluR1 levels were determined by biotin labeling. REMD mice exhibited less surface GluR1 than that of the SDC group and the decrease in GluR1 level by REMD was reversed after leptin treatment. (***) $P < 0.001$ vs. SDC, (###) $P < 0.001$ vs. REMD.

($F_{(3,24)} = 0.278$, $P > 0.5$), moving velocity ($F_{(3,24)} = 1.71$, $P > 0.1$), and body weight ($F_{(3,24)} = 0.313$, $P > 0.5$) (Supplemental Fig. 1).

Leptin restores REMD-induced decrease in surface expression of GluR1

We determined whether surface expression of the GluR1 subunit of AMPA receptor was affected by REMD. Mice were randomly assigned to naïve, unpaired REMD, and SDC groups. Basolateral amygdalar (BLA) tissues were dissected out and surface receptors were labeled with biotin 1 h after REMD. Biotinylated receptors were precipitated and surface GluR1 was determined by immunoblotting. Figure 3A shows that the GluR1 level in the SDC group ($149.9 \pm 21.1\%$ of naïve, $n = 5$) was significantly higher compared to that in the naïve group ($F_{(3,16)} = 6.451$, $P < 0.01$). REMD reversed the conditioning-induced increase in GluR1 to $75.6 \pm 7.7\%$ ($n = 5$) of naïve control. In separate groups, mice were conditioned, followed by intraperitoneal injection of saline or 4 mg/kg leptin before undergoing REM deprivation. As illustrated in Figure 3B, REMD mice exhibited less surface GluR1 level ($73.3 \pm 5.0\%$ of SDC, $n = 13$, $P < 0.001$) which could be restored by leptin treatment to $106.6 \pm 5.9\%$ of SDC ($n = 14$). No differences in the immunoreactivities against whole-cell lysate of GluR1 level ($F_{(2,54)} = 1.498$, $P > 0.1$) were detected in SDC, REMD, and leptin groups, suggesting that the total GluR1 level was not affected by REMD.

Leptin restores REMD-induced decrease in AMPA/NMDA ratio

Mice were randomly assigned to naïve, unpaired, REMD, and SDC groups. Amygdalar slices were made 1 h after REMD and whole-cell recordings were made from the soma of visually identified pyramidal-like neurons located in the LA. Neurons were identified

as projection neurons based on their morphology. We determined whether the excitatory synaptic transmission in the LA neurons was altered after fear conditioning by measuring the relative contribution of AMPA receptor and NMDA receptor (NMDAR) to the EPSCs which could minimize the effect of slice-to-slice variability and has been proven as a sensitive assay for detecting differences in the glutamatergic synaptic strength (Ungless et al. 2001; Bellone and Luscher 2006; Clem and Barth 2006; Kourrich et al. 2007). AMPAR-mediated EPSC was evoked when the neurons were voltage-clamped at -70 mV, whereas NMDAR-mediated EPSC was determined as current amplitude at 50 msec after peak EPSC amplitude at a holding potential of $+40$ mV (Du et al. 2008). In naïve and unpaired mice, the AMPA/NMDA ratios were 0.98 ± 0.07 ($n = 11$) and 1.00 ± 0.08 ($n = 12$), respectively. The ratio was significantly higher in the SDC rats (1.45 ± 0.15 , $n = 13$, $P < 0.05$ vs. naïve and unpaired mice) (Fig. 4). REMD abrogated the conditioning-induced increase in AMPA/NMDA ratio to the level of unpaired (0.83 ± 0.08 , $n = 9$, $P < 0.01$ vs. SDC and leptin mice). Leptin treatment restored the AMPA/NMDA ratio to 1.30 ± 0.09 ($n = 12$). We performed experiments to record EPSC before and after the application of leptin. Consistent with the report in the hippocampus (Moult et al. 2010), leptin increased the amplitude of EPSC to $165.0 \pm 25.7\%$ of baseline ($n = 6$, one neuron per mouse) (Supplemental Fig. 2).

One-way ANOVA for mEPSC amplitude showed a significant effect of group ($F_{(4,42)} = 5.092$, $P < 0.05$), and Newman-Keuls post hoc tests revealed that the SDC (13.9 ± 1.4 pA, $n = 9$) differed from the unpaired (10.2 ± 0.5 pA, $n = 12$, $P < 0.01$) group (Fig. 5A). REMD abrogated the conditioning-induced increase in amplitude of mEPSCs (9.1 ± 0.5 pA, $n = 9$), which was reversed by leptin treatment (12.5 ± 1.0 pA, $n = 7$, $P < 0.05$) (Fig. 5B). We found similar results for mEPSC frequency. There was a significant effect of group ($F_{(4,42)} = 7.679$, $P < 0.01$), and Newman-Keuls post hoc tests revealed that the SDC (22.9 ± 1.7 Hz, $n = 9$) differed from the unpaired (16.2 ± 0.9 Hz, $n = 12$, $P < 0.001$) group (Fig. 5). REMD abrogated the conditioning-induced increase in amplitude of mEPSCs (14.1 ± 0.4 Hz, $n = 9$), which was reversed by leptin treatment (17.7 ± 1.7 Hz, $n = 7$, $P < 0.05$) (Fig. 5C).

Paired-pulse facilitation (PPF) is a measure of short-term plasticity widely used to probe for changes in presynaptic function

Figure 4. Effects of REMD and leptin on the AMPA/NMDA ratio at the thalamo-LA synapses. Plot of AMPA/NMDA ratio in the naïve ($n = 11$, five mice), unpaired ($n = 12$, five mice), SDC ($n = 14$, six mice), REMD ($n = 9$, four mice), and leptin (4 mg/kg, $n = 12$, five mice)-treated mice. AMPAR-mediated EPSC was evoked when the neurons were voltage-clamped at -70 mV, whereas NMDAR-mediated EPSC was determined as current amplitude at 50 msec after peak EPSC amplitude at a holding potential of $+40$ mV (Du et al. 2008). Sample traces were the average of 3–5 successive responses. (*) $P < 0.05$ vs. unpaired and naïve mice, (##) $P < 0.01$ vs. SDC and leptin-treated mice.

Figure 5. Effects of REMD and leptin on the amplitude and frequency of mEPSC at the thalamo-LA synapses. (A) Sample traces of mEPSCs taken from slices of the naïve ($n = 10$, five mice), unpaired ($n = 11$, five mice), SDC ($n = 9$, six mice), REMD ($n = 9$, six mice), and leptin (4 mg/kg, $n = 9$, six mice)-treated mice. mEPSCs were recorded in the LA neurons at a holding potential of -70 mV in the presence of tetrodotoxin (TTX) ($1 \mu\text{M}$). (B,C) Summary plots of the amplitude (B) and frequency (C) of mEPSC in the naïve, unpaired, SDC, REMD, and leptin-treated mice. (***) $P < 0.001$ vs. unpaired and naïve, (#) $P < 0.05$ vs. REMD.

because changes in PPF are inversely related to neurotransmitter release. To determine whether the increased synaptic strength involved a presynaptic mechanism, we analyzed PPF in slices from the naïve, unpaired, paired, SDC, and REMD mice. Ratios of the amplitude of the second EPSC to the amplitude of the first EPSC were examined at different interpulse intervals (Fig. 6). PPFs at 30- and 50-msec intervals in the SDC group were significantly less (30 msec, 0.73 ± 0.06 ; 50 msec, 0.81 ± 0.07) than those of naïve (30 msec, 1.42 ± 0.11 ; 50 msec, 1.20 ± 0.13) and unpaired (30 msec, 1.55 ± 0.15 ; 50 msec, 1.32 ± 0.16) mice (30 msec, $F_{(4,67)} = 13.58$, $P < 0.001$; 50 msec, $F_{(4,67)} = 6.74$, $P < 0.001$). REMD reversed conditioning-induced decreases in PPF to the levels of unpaired group (30 msec, 1.31 ± 0.08 ; 50 msec, 1.20 ± 0.07). This result was consistent with a previous report that enhanced synaptic efficacy after fear conditioning was mediated, at least in part, by an increase in presynaptic release probability (Lin et al. 2010). However, it was noted that leptin had no effect on the PPF, ruling out the presynaptic site of action.

Leptin restores REMD-induced reduction of PTEN expression

Previous studies have shown that leptin promoted GluR1 trafficking via inhibition of PTEN (Moult et al. 2010), a dual protein/lipid phosphatase which dephosphorylates the D3 position of PI-3 kinase product phosphatidylinositol 3,4,5-triphosphate (PIP₃), and consequently down-regulates the effect of the PI-3 kinase pathway (Maehama and Dixon 1998). We determined whether leptin restored fear memory through phosphorylation of PTEN (inactivation) and Akt (activation of PI-3 kinase). Mice were randomly assigned to naïve, unpaired, SDC, and REMD. Phosphorylated states of PTEN (p-PTEN) and Akt from the BLA were quantified in each group 1 h after REMD. Figure 7A shows that the ratio of p-PTEN/PTEN level in the SDC group ($128.3 \pm 8.2\%$ of naïve, $n = 5$) was significantly higher compared to those in naïve and

unpaired groups ($F_{(3,21)} = 7.647$, $P < 0.01$). REMD reversed the conditioning-induced increase in p-PTEN/PTEN ratio to $88.2 \pm 6.8\%$ ($n = 6$) of naïve control. In separate groups, mice were conditioned, followed by intraperitoneal injection of saline or 4 mg/kg leptin before undergoing REM deprivation. As illustrated in Figure 7B, leptin reversed the REMD-induced decrease in p-PTEN/PTEN ratio to $114.3 \pm 3.3\%$ ($n = 4$) of SDC group.

As shown in Figure 7A, the ratio of pAkt/Akt level in the SDC group was significantly higher compared to those in naïve and unpaired groups ($F_{(3,21)} = 7.423$, $P < 0.01$). REMD reversed the conditioning-induced increase in pAkt/Akt ratio to $73.7 \pm 6.9\%$ ($n = 6$) of naïve control. Leptin reversed the REMD-induced decrease in pAkt/Akt ratio to $106.3 \pm 3.6\%$ ($n = 6$) of SDC group (Fig. 7B).

It has been shown that leptin activated PI-3K/Akt leading to phosphorylation of GSK-3 β on Ser9 and thereby inhibited its activity (Garza et al. 2012). We determined whether leptin restored fear memory through phosphorylation of GSK-3 β . As shown in Figure 7A, the ratio of pSer9 GSK-3 β /GSK-3 β level in the SDC group was significantly higher compared to those in naïve and unpaired groups ($F_{(3,21)} = 7.931$, $P < 0.01$). REMD reversed the conditioning-induced increase in pSer9 GSK-3 β /GSK-3 β ratio to $77.7 \pm 4.2\%$ ($n = 6$) of naïve control. Leptin reversed the REMD-induced decrease in pSer9 GSK-3 β /GSK-3 β ratio to $130.7 \pm 12.6\%$ ($n = 7$) of SDC group (Fig. 7B).

Inhibition of protein tyrosine phosphatase by bisperoxovanadium prevents REMD-induced cognitive deficits

Bisperoxovanadium (bpV) is a small-molecule protein tyrosine phosphatase inhibitor that specifically and potently inhibits PTEN activity in vitro (Schmid et al. 2004). Administration of bis-

Figure 6. Effects of REMD and leptin on the PPF of EPSC at the thalamo-LA synapses. (A) Sample traces of PPF of EPSCs taken from slices of the naïve ($n = 17$, five mice), unpaired ($n = 12$, five mice), SDC ($n = 12$, five mice), REMD ($n = 14$, five mice), and leptin (4 mg/kg, $n = 13$, five mice)-treated mice. Sample traces were the average of 3–5 successive responses. (B) Plot of PPF in the naïve, unpaired, SDC, REMD, and leptin-treated mice. (***) $P < 0.001$ vs. other groups.

Figure 7. Effects of REMD and leptin on the phosphorylated states of PTEN, Akt, and GSK-3 β . (A) Mice were given five pairings of tone and footshock. REM sleep was prevented in a 24-h period 1 h after the training. Phosphorylated PTEN, Akt, and GSK-3 β were measured at 1 h after REMD. (*) $P < 0.05$, (**) $P < 0.01$ vs. naive mice. (#) $P < 0.05$, (##) $P < 0.01$ vs. SDC mice. (B) Mice received five tone-footshock pairings and were injected intraperitoneally with 4 mg/kg leptin before REMD. Phosphorylated PTEN, Akt, and GSK-3 β were measured at 1 h after REMD. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs. SDC mice. (##) $P < 0.01$, (###) $P < 0.001$ vs. REMD mice.

peroxovanadium promoted neuroprotection in various CNS injury models through activation of the PI-3K/Akt pathway (Zhang et al. 2007; Song et al. 2010; Sury et al. 2011; Walker et al. 2012). To determine the functional effects of PTEN inhibition, the effects of bpV on REMD-induced cognitive deficits were examined.

Mice received five tone-footshock pairings and were injected intraperitoneally with saline, 40 μ g (bpV1), 80 μ g (bpV2), 160 μ g (bpV3), or 400 μ g/kg bpV (bpV4) before REMD. Retention of memory was tested 1 h after REMD. As shown in Figure 8A, both saline and bpV-treated groups were able to acquire tone-shock association to the equivalent levels ($F_{(4,37)} = 0.964$, $P > 0.1$). However, when tested 1-h REMD, mice in the saline group ($10.8 \pm 3.2\%$, $n = 11$) displayed a significantly less cue freezing response than that of the bpV-treated group ($F_{(4,37)} = 18.71$, $P < 0.001$) (Fig. 8B). Similarly, saline group ($11.8 \pm 3.5\%$, $n = 11$) also exhibited a lower contextual freezing response compared to that of the bpV-treated group ($F_{(4,37)} = 6.196$, $P < 0.001$) (Fig. 8C). These results suggest that pretreatment with bpV prevents REMD-induced cognitive deficits.

We determined whether leptin altered the effect of PTEN inhibition on REMD-induced impairment of fear memory. Mice received five tone-footshock pairings and were injected intraperitoneally with saline, 4 mg/kg leptin, or 4 mg/kg leptin + 400 μ g/kg bpV before REMD. Retention of memory was

sleep-dependent memory processing (Graves et al. 2001; Louie and Wilson 2001; Ribeiro et al. 2002). Here, we confirmed this assumption by showing that REMD immediately after conditioning impaired both cue and contextual fear memory. The impairment of cue-associated memory was accompanied by a decrease in

tested 1 h after REMD. Figure 8D shows that mice in the saline group ($10.8 \pm 3.2\%$, $n = 11$) displayed a significantly lower cued freezing response compared to those in leptin and leptin + bpV groups ($F_{(2,32)} = 58.12$, $P < 0.001$). Similarly, the saline group ($11.8 \pm 3.5\%$, $n = 11$) also exhibited a lower contextual freezing response compared to those in leptin and leptin + bpV groups ($F_{(2,44)} = 15.93$, $P < 0.001$) (Fig. 8E). Importantly, there was no difference between leptin and leptin + bpV groups in both cued ($t_{(22)} = 0.723$, $P > 0.1$) and contextual ($t_{(34)} = 1.008$, $P > 0.1$) freezing responses. These results suggest that leptin occludes the effect of bpV.

Discussion

It has been suggested that reprocessing of newly acquired material within the neural networks during sleep significantly contributes to the formation of different types of memory (Wilson and McNaughton 1994; Hirase 2001; Louie and Wilson 2001; Hoffman and McNaughton 2002). Recent studies have shown that the hippocampus and amygdala may be the platform for

Figure 8. Bisphenoxovanadium prevents REMD-induced cognitive deficits. (A) Mice received five tone-footshock pairings and were injected intraperitoneally with saline, 40 μ g (bpV1), 80 μ g (bpV2), 160 μ g (bpV3), or 400 μ g/kg bpV (bpV4) before REMD. All groups of mice were able to acquire tone-shock association. (B) Twenty-four hours after conditioning, the mice were tested for memory retention in a new chamber with CS. The freezing responses in the bpV-treated groups were significantly higher compared to that in the saline group. (***) $P < 0.001$ vs. saline group. (C) Twenty-four hours after conditioning, the mice were tested for memory retention in the conditioning chamber without CS. The freezing responses in the bpV-treated groups were significantly higher compared to that in the saline group. (***) $P < 0.001$ vs. saline group. (D, E) Mice received five tone-footshock pairings and were injected intraperitoneally with saline, 4 mg/kg leptin, or 4 mg/kg leptin + 400 μ g/kg bpV before REMD. Cued (D) and contextual (E) freezing responses were assessed 1 h after REMD.

surface expression of GluR1, a decrease in both frequency and amplitude of mEPSCs, and an increase in PPF. These results suggest that inhibition of enhanced synaptic efficacy after fear conditioning by REMD is mediated by both presynaptic and postsynaptic mechanisms. Leptin treatment rescued memory deficits induced by REMD. Leptin reversed a REMD-induced decrease in p-PTEN/PTEN, pAkt/Akt, and pSer9 GSK-3 β /GSK-3 β ratios. Furthermore, a tyrosine phosphatase inhibitor, bpV, mimicked the effect of leptin by preventing REMD-induced cognitive deficits.

We have shown previously that fear conditioning resulted in a decrease in PPF (Lin et al. 2010), suggesting that enhanced synaptic efficacy after fear learning was mediated, at least in part, by increased presynaptic transmitter release. Here we demonstrate that REMD after fear conditioning restored PPF to naïve and unpaired levels. Accumulating evidence indicates that sleep deprivation increases astrocyte release of ATP (Florian et al. 2011) which is rapidly hydrolyzed to adenosine via a variety of ectonucleotidases (Dunwiddie et al. 1997). Adenosine acting on adenosine A1 receptors may inhibit amygdalar neurons by reducing transmitter release presynaptically. In agreement with this hypothesis, we found that REMD reversed a conditioning-induced decrease in PPF (Fig. 7). Interestingly, leptin treatment reversed a REMD-induced decrease in surface expression of GluR1 and AMPAR/NMDAR ratio without affecting REMD's effect on PPF. These results suggest that leptin exerted its effects predominantly on the postsynaptic sites.

Fear conditioning also increased surface expression of GluR1 and AMPA/NMDA ratio, suggesting a postsynaptic increase in AMPA receptors (Lin et al. 2010). REMD reversed these effects of fear conditioning. These results are consistent with the recent report in the hippocampus that REMD decreased synaptic transmission and long-term potentiation in dorsal CA1 that was associated with a decrease in GluR1 (Ravassard et al. 2009).

The finding that leptin prevents the effects of REMD on cognitive abilities is in agreement with previous reports showing the beneficial effects of leptin on synaptic plasticity and learning and memory (Beccano-Kelly and Harvey 2012). For example, administration of leptin into the hippocampus enhanced spatial memory tasks, increased the magnitude of LTP, and promoted the conversion of short-term potentiation into LTP (Shanley et al. 2001; Wayner et al. 2004). We examined the effects of leptin on the cell surface expression of GluR1 in the BLA and found that leptin restored REMD-induced decreases in GluR1 to the level of the SDC.

Previous studies have demonstrated that PI-3 kinase plays an important role for the action of leptin in the hypothalamus, brainstem, and hippocampus. In the arcuate nucleus, leptin depolarizes arcuate proopiomelanocortin (POMC) and ventral premammillary nucleus (PMV) neurons via a PI3K-dependent activation of a putative canonical transient receptor potential (TRPC) channel (Hill et al. 2008; Qiu et al. 2010; Williams et al. 2011). On the other hand, leptin hyperpolarizes arcuate NPY/AgRP neurons via a PI3K-dependent activation of a K_{ATP} channel (Spanswick et al. 1997; van den Top et al. 2004). In adult hippocampal slices, leptin increased GluR1 surface expression which was correlated with enhanced PI3K-driven PtdIns(3,4,5),P3 immunostaining (Moult et al. 2010). We found that leptin increased p-PTEN/PTEN, pAkt/Akt, and pSer9 GSK-3 β /GSK-3 β ratios. It is conceivable that leptin promotes phosphorylation and inactivates PTEN leading to activation of PI-3K/Akt which drives phosphorylation and inactivation of GSK-3 β and subsequently synaptic GluR1 insertion.

The endocrine hormone leptin enters the brain via transport across the brain–blood barrier. Previous studies in mice showed that an intraperitoneal dose of 6 mg/kg leptin produced a plasma concentration of 15,000 ng/mL after 1 h which decreased to 2000

ng/mL in the second hour (Enriori et al. 2011). Serum leptin concentration at 2 h after intraperitoneal injection of 1.5 mg/kg was around 150 ng/mL (Harris et al. 2003). In the present study, the effective doses we used were 2–4 mg/kg which could produce plasma concentrations between 150 and 2000 mg/mL. Leptin at these doses has been shown to decrease infarct volume (Zhang et al. 2007) and activate neuroprotective signals in mouse cerebral cortex (Valerio et al. 2006). Accumulating evidence indicates a potential cognitive enhancing effect for leptin as it regulates a number of hippocampal synaptic activities that underlie learning and memory. In the present study, we have demonstrated that intraperitoneal injection of leptin to REM sleep-deprived mice rescued cue fear memory impairment. The beneficial effect of leptin at the cellular level may be attributed to its ability to inhibit PTEN resulting in the phosphorylation of PI3K and GSK3 β which in turn promotes GluR1 surface expression in the amygdala. However, the present study did not prove directly that the leptin-driven effects are due to activation of leptin receptors located in the amygdala. In the absence of good selective leptin receptor antagonists, future study needs to perform experiments in leptin-insensitive (*db/db*) mice to clarify this.

Materials and Methods

Animals

Male C57BL/6 mice weighing 20–25 g were used. They were housed in group cages of four mice each in an air-conditioned vivarium with free access to food and water. Throughout the study, a 12:12-h light–dark cycle was maintained with light on at 8 a.m. Behavioral tests were performed in the light phase. All procedures adhered to the Guidelines for Care and Use of Experimental Animals of the National Cheng-Kung University. Recombinant mouse leptin was purchased from Sigma. The stock solution of leptin was prepared according to the manufacturer's recommendation. The amounts of leptin needed were then prepared by diluting the stock solution with saline, bringing the total volume to 0.5 mL. Leptin solution was then injected intraperitoneally at the indicated time. The vehicle-treated mice were injected intraperitoneally with 0.5 mL of saline.

Fear conditioning

Fear conditioning occurred in a 30- × 24- × 21-cm operant chamber (Med Associates). The chamber was equipped with a shock floor, house light, and speaker mounted on the wall through which tone presentations were delivered. The shock floor, consisting of stainless-steel rods, was wired to a shock generator for footshock delivery. A house light provided illumination during all sessions. All were controlled by FreezeScan software (Clever Systems). The chamber was cleaned with 75% ethanol before each mouse was trained or tested for contextual fear conditioning. On the first day of training, mice were transported in their home cage to a behavioral room. After a 60-min habituation period in the room, mice were placed in the training chamber for 120 sec. After the acclimation period, mice were presented with a pure tone (20 sec, 3 kHz) that co-terminated with footshock (3 sec, 0.85 mA). This tone–footshock pairing procedure was repeated five times with an inter-trial interval (ITI) of 60 sec. After the last tone paired shock delivery, mice were allowed to explore the context for 1 min before removal from the chamber. Unpaired mice received the same number of CS and US but in an unpaired pseudorandom fashion. The leptin groups were injected intraperitoneally with 1, 2, or 4 mg/kg leptin 30 min after fear conditioning (30 min before REMD).

Twenty-four hours after training, mice were returned to the training chamber for 3 min without exposure to the tone or footshock for a context fear test. At the end of the contextual test, mice were returned to their home cage. Approximately 1 h later, mice were placed in a novel context for a 120-sec baseline followed by 3 min of the tone to assess cue-dependent fear conditioning.

The novel context was altered on an opaque Plexiglas box. Specifically, grid floors were replaced with smooth Plexiglas floors and the chamber was cleaned with 1% acetic acid before each mouse was tested. The behavior of mice was recorded by video camera mounted above the conditioning chamber. Freezing was defined as the absence of any movement except for respiration and measured automatically using FreezeScan software. Freezing data are presented as percent time spent freezing. In order to evaluate auditory retention specifically, pretone freezing was subtracted from freezing that occurred during the tone period.

REM sleep deprivation

Deprivation of REM sleep was accomplished by using the “flower pot” technique adapted and modified from Boutrel et al. (1999). Mice were placed on a Plexiglas pedestal (REMD, 3 cm in diameter; SDC, 7.5 cm in diameter) surrounded by water. The base of the pot extended above the level of the water by ~1 cm. The floor was covered with water 12 cm deep. Food and water were available ad libitum. In this situation, the mice were unable to completely relax the large muscle groups without falling from the platform, getting wet, and waking.

Slice preparation

Mice were decapitated and their brains rapidly removed and placed in cold oxygenated artificial cerebrospinal fluid (ACSF) solution. Subsequently, the brain was hemisected and cut transversely posterior to the first branch and anterior to the last branch of the superior cerebral vein. The resulting section was glued to the chuck of a Vibroslice tissue slicer. Transverse slices of 300 μ m thickness were cut and the appropriate slices placed in a beaker of oxygenated ACSF at room temperature for at least 1 h before recording. ACSF solution had the following composition (in mM): NaCl 117, KCl 4.7, CaCl_2 2.5, MgCl_2 1.2, NaHCO_3 25, NaH_2PO_4 1.2, and glucose 11. The ACSF was bubbled continuously with 95% O_2 –5% CO_2 and had a pH of 7.4.

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were made from the LA neurons. EPSCs were evoked at 0.05 Hz by extracellular stimulation of fibers emerging from the internal capsule which originate in the medial geniculate nucleus of the thalamus and project monosynaptically to the LA using a bipolar electrode. Patch electrodes were pulled from a thick-walled glass capillary (0.86 mm i.d., 1.5 mm o.d.) to a tip resistance of 3–5 M Ω . The composition of the internal solution for recording neuronal properties and action potentials was (in mM): K-gluconate 140, KCl 10, EGTA 1, phosphocreatine 10, Mg-ATP 4, Na-GTP 0.3, and HEPES 10. The composition of the internal solution for recording AMPA/NMDA ratios was (in mM): cesium methane-sulfonate 128, NaCl 20, EGTA 1, CaCl_2 0.3, MgCl_2 1, Na-ATP 3, Na-GTP 0.4, and HEPES 10. The final pH of the internal solution was adjusted to 7.3 by adding 1 M KOH or CsOH; the final osmolality was adjusted to 290–300 mOsm by adding sucrose. Records were low-pass-filtered at 2.5–20 kHz and digitized at 5–50 kHz. The signal was monitored and recorded with an Axopatch 200B amplifier. On-line analysis and control of experimental acquisition was accomplished via a 586 (Intel)-based PC clone and a Digidata 1320 computer interface. AMPAR-mediated EPSC was evoked when the neurons were voltage-clamped at –70 mV, whereas NMDAR-mediated EPSC was determined as current amplitude at 50 msec after peak EPSC amplitude at a holding potential of +40 mV (Du et al. 2008). AMPA/NMDA ratios were also measured by recording EPSCs at +40 mV before and after application of the NMDAR blocker D-APV (50 μ M). NMDAR-mediated EPSC was calculated by subtracting the response in the presence of D-APV from the control response without adding D-APV. The peak of the AMPA EPSC was divided by the peak of the NMDA EPSC to yield an AMPA/NMDA ratio (Ungless et al. 2001). Events of mEPSCs were sampled at 10 kHz and filtered at 5 kHz using Axograph X. Tetrodotoxin (0.5 μ M) and bicuculline (10 μ M) were added to the bath.

Western blot analysis

One hour after test, mice were sacrificed by decapitation. LA and BLA were sonicated briefly in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μ g/mL leupeptin, and 4 μ g/mL aprotinin). Following sonication, the samples were centrifuged at 7500 rpm for 15 min and the supernatant was obtained after pelleting the crude membrane fraction by centrifugation at 50,000 rpm for 1 h at 4°C. Protein concentration in the soluble fraction was then measured using a Bradford assay, with bovine serum albumin as the standard. Equivalent amounts of protein for each sample were resolved in 8.5% SDS–polyacrylamide gels, blotted electrophoretically to Immobilon, and blocked overnight in TBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 3% bovine serum albumin. For detection of the protein level of PTEN and phosphorylated forms of Akt and MAPK, blots were incubated with anti-PTEN (1:2500, Santa Cruz Biotechnology), anti-phospho-Akt (1:2500, Santa Cruz Biotechnology), and anti-phospho-ERK (1:2500, Santa Cruz Biotechnology) antibodies. An enhanced chemiluminescence kit (NEN Life Science Products) was used for detection. Western blots were developed in the linear range used for densitometry. The density of the immunoblots was determined by an image analysis system installed with the software BIO-1D (Vilber Lourmat).

Statistical analysis

Data were expressed as mean $\pm$ SEM. Behavioral data were analyzed by ANOVA. Newman–Keuls multiple comparison tests were used as post hoc comparisons. Western blotting and electrophysiological data were analyzed by Student's *t* tests. The level of significance was $P < 0.05$.

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The beneficial effects of leptin on REM sleep deprivation-induced cognitive deficits in mice

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